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THE JOURNAL OF PHYSIOLOGY

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BY

L. E. BAYLISS	G. L. BROWN	E. G. T. LIDDELL
H. S. RAPER	E. B. VERNEY (<i>Chairman</i>)	F. R. WINTON

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POTASSIUM ACCUMULATION IN MUSCLE AND ASSOCIATED CHANGES¹

BY P. J. BOYLE AND E. J. CONWAY

*From the Department of Biochemistry,
University College, Dublin*

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WE are concerned here chiefly with an account of the mechanism underlying the accumulation of potassium in the excised sartorius of the frog. This accumulation may, under suitable conditions, be very great, the cation entering against a gradient, and to upwards of three times the normal content.

The relations deduced predict with surprising accuracy the mean changes in potassium, in the smaller permeating anions (e.g. chloride) and in the cell volume—the latter to within a few per cent. with change in 'fibre water' up to 100%.

Such processes have, as we think, a general application to the cellular accumulation of potassium. It will, in fact, appear that with the membrane as considered, accumulation of potassium in the concentrations found should go *pari passu* with the normal growth of the cell, so that there is no need to make any further assumptions in the way of special processes.

A necessary association appears between potassium and the phosphorylated compounds important for the carbohydrate cycle, and such a relation has been already experimentally observed [e.g. Myers & Fine, 1913; Verzár & Somogyi, 1939; Myers & Manguin, 1940].

The theoretical treatment too affords an exact quantitative interpretation of the resting potentials in muscle.

It is shown that the membrane of the muscle fibre in the excised sartorius of the frog, when maintained under suitable conditions, is permeable to potassium and cations of the same or smaller diameter,

¹ A preliminary account of the present paper has already appeared in a letter to *Nature* [Conway & Boyle, 1939].

but impermeable to sodium or larger cations. It is at the same time permeable to the smaller anions such as chloride, bicarbonate, etc., but not to larger anions of the type of phosphocreatine, adenylypyrophosphoric acid, etc.

While it is now generally accepted that the excised muscle is quite permeable to the potassium ion, it has also been generally believed to be essentially impermeable to anions [e.g. Höber, 1922; Fenn, 1936; Eggleton, Eggleton & Hamilton, 1937], though Fenn [1936] has recognized and emphasized certain difficulties arising from such a view and considered the position unsatisfactory.

As may be seen from the results described there can be no doubt that chloride enters the fibre freely when the potassium concentration outside is raised to 12 m.equiv./l. From that concentration up to and beyond 210 m.equiv./l. potassium is greatly concentrated in muscle without change in volume—provided the conditions are correctly adjusted—and chloride enters in equivalent relation. So far from the differential permeability of the membrane being impaired it is improved, since sodium is better excluded and the normally non-penetrating anions within are better held. The impermeability to sodium is usually perfectly maintained over 24 hr. at about 2–3° C., whereas without the raised potassium sodium enters very appreciably.

The reason for the improved conditions with raised potassium is apparently associated with the lowering of the potential gradient across the membrane. For the excised muscle this would seem too high for the maintenance of the normal equilibrium and the permeability alters towards a new equilibrium characterized by an inrush of chloride, exchanging partly with anions inside and being in part accompanied by sodium while potassium is lost.

The main evidence for the anion impermeable view has been the apparent similarity of the chloride in the muscle interspaces with the total chloride in muscle; also the nature of the resting potentials and the effect of varying contact fluids. It has been shown in a previous paper [Boyle, Conway, Kane & O'Reilly, 1941] that only 1–2 % of the external chloride is required within the fibres with a chloride permeable membrane, and it is obvious that this could not be successfully differentiated from the ten times greater quantity in the interspaces, with the methods hitherto used for determining the spaces.

As to the electrical findings, so far from these being against the view that the muscle fibre is impermeable to anions, it is only by accepting this that they can be quantitatively interpreted.

The manner in which the permeability of the excised muscle may differ from that of the muscle in vivo is discussed at the end in the light of the recent findings concerning injections of radio-potassium, radio-sodium and phosphate [e.g. Greenberg, Joseph, Cohn & Tufts, 1938; Joseph, Cohn & Greenberg, 1939; Hahn, Hevesey & Rebbe, 1939].

I. METHODS

Sartorius muscles from *Rana temporaria* were used throughout, unless otherwise stated. The tissues were excised immediately after killing the frog. For each observation single muscles from four frogs were dried rapidly between filter paper, transferred to a tared watch-glass with cover, quickly weighed and transferred to the immersion fluid. The companion tissues after drying and weighing were analysed directly.

In the short-period experiments, ranging from a few minutes to several hours, the muscles were immersed in 30 ml. fluid contained in wide glass tubes. They were stirred by a current of gas bubbling through; the gas mixture being 3% carbon dioxide with 97% oxygen when solutions of the Barkan type [Barkan, Broemser & Hahn, 1921] were used, or oxygen with the original Ringer or Ringer-Locke solutions.

For long-period immersions at low temperatures, ranging from 16 to 144 hr., the solution contained in small conical flasks was first prepared by bubbling the gas through for 30 min. and then transferring the well-stoppered flasks to the refrigerator (at 2-3° C.) for 1 hr. The muscles were then immersed and the stoppered flasks replaced in the refrigerator.

Immersion fluids. The immersion fluid mostly used was similar to the Barkan modification of Ringer solution. The pH was maintained at 7.3 by bubbling 3% carbon dioxide in oxygen through the mixture as described below. This solution was chosen, although it contains much less bicarbonate than the frog's plasma, because with a low bicarbonate-chloride ratio, the chloride gives a good measure of the total diffusible anions, even if the bicarbonate behaves somewhat differently from the chloride.

The Barkan fluid had the following constant composition, apart from varying sodium and potassium chloride additions:

NaHCO ₃	...	11.9 mM./l.	(40 ml. of 2.5%)
Na ₂ HPO ₄	...	0.67 "	(5 ml. M/7.5)
NaH ₂ PO ₄	...	0.10 "	(5 ml. M/50)
CaCl ₂	...	1.8 "	(10 ml. 2%)
Glucose	...	3.9 "	(0.7 g.)

The primary and secondary phosphates were included in the one stock solution.

Another fluid occasionally used simulated as closely as possible the electrolyte composition of plasma with glucose added to make it isosmotic with excised muscle. It had the following composition (mM./l.):

Sodium ion	103.8	Bicarbonate ion	25.0
Potassium ion	2.5	Phosphate (P) ion	3.0
Calcium ion	0.9	Sulphate (SO ₄) ion	1.8
Magnesium ion	1.2	Gluconate ion	1.9
Chloride ion	74.5	Glucose	26.0

The Na⁺, K⁺, Cl⁻, HCO₃⁻ and P values correspond to the data for frog's plasma assembled by Fenn [1936] from his own and other analyses. The calcium is about half the total concentration found and therefore represents approximately the expected value for the ionized calcium. The sulphate and magnesium concentrations correspond to data obtained here [Conway & Kane, 1934 a, b; Cruess-Callaghan, 1935].

The gluconate ion is additional with respect to plasma composition but is present in minute amounts only, and the glucose is also much higher than normal blood values, being

added for osmotic equilibrium with muscle. The immersion fluid (apart from glucose) may be made up from the following stock solutions:

- (I) 21.2% NaCl.
- (II) 5.25% NaHCO_3 ,
0.89% Na_2HPO_4 .
- (III) 1.70% KH_2PO_4 ,
3.70% KCl,
7.39% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,
2.30% Na_2SO_4 (anhydrous).
- (IV) 10% $\text{Ca}(\text{C}_6\text{H}_{10}\text{O}_7)_2$ (gluconate).

To make 500 ml., 10 ml. of (I) is introduced into a 500 ml. flask, 20 ml. of (II) added, the solution diluted to near the full volume, 2 ml. of (III) and 2 ml. of (IV) introduced and the mixture made to the mark with water. The solution is saturated by a 3% carbon dioxide and 97% oxygen mixture which is bubbled through.

Sodium and potassium. These were determined as described in the previous paper.

Magnesium. This was determined by the hydroxyquinoline method using the modification of G. Cruess-Callaghan [1935].

Chloride was measured by a micro-diffusion method [Conway, 1935].

Measurements of resting potentials. The electrodes and modes of contact are described in the text. The actual readings were made on a Cambridge potentiometer with Weston standard cell, and using a galvanometer sensitive to less than 0.5 mV. The measurements were made on fresh muscle immediately after excision, or in special instances after washing for half an hour in isotonic glucose.

Cryoscopic. Freezing-points of fluids as mentioned in the text were carried out with the usual Beckmann technique, there being no volume restriction. Precautions were taken against excessive supercooling and the measurements were taken as accurate to 0.01° C.

Calculation of concentrations in the 'fibre water'. In accordance with the previous paper [Boyle *et al.* 1941] such concentrations are calculated by the equation

$$C_f = (C_m - 0.13C_o) / 0.67,$$

where C_f is mM./l. of 'fibre water', C_m mM./kg. muscle and C_o mM./l. of external fluid. For substances such as adenylypyrophosphate, carnosine, etc., in these experiments, C_o is zero.

II. RESULTS

Concerning electrolyte permeability of the excised sartorius

Provided the concentration ratio of the diffusible ion across the membrane is below a certain level the excised sartorius shows a very definite type of electrolyte permeability.

From our results Table 1 indicates the general nature of the permeability with respect to sodium, potassium and chloride.

The table shows the results of 2 hr. immersions at room temperature (with stirring by gas bubbling as described under Methods) and of immersions over 24 hr. at low temperature. (2–3° C.).

The experiments at low temperature with increasing external potassium were designed (from principles to be described later) to produce mean weight increases ranging from 8 to 10%, and from such the actual figures differ but little.

TABLE I

TABLE I

Type of solution	Na, K and Cl conc. in external fluid m.equiv./l.			Change in composition of muscle m.equiv./kg. muscle			Wt. after/before	Na and Cl conc. in muscle g./100 g.	
	Na	K	Cl	Na	K	Cl		g./1 ml. ext. sol.	
								Na	Cl
Immersions for 2 hr. at room temperature									
Locke	131	1.3	137	35 (5)	—	31 (5)	1.05 (5)	50	30
A	133	1.3	124	30	-18	—	1.00	41	—
B	104	2.5	74	10 (2)	-11 (2)	9 (2)	1.01 (2)	33	27
A	86	30	106	8 (6)	—	—	1.05 (6)	38	—
A	70	80	149	1 (5)	34 (5)	—	0.96 (5)	34	48
Immersions for 24 hr. at 2-3° C.									
A	86	18	94	11	7.4 (2)	14	1.13 (4)	38	26
A	86	30	108	-3 (4)	19 (4)	18	1.10 (6)	23	27
A	86	90	166	-3 (3)	59	78	1.04 (4)	23	52
A	86	120	198	-3	104	99	1.04 (2)	22	50
A	86	150	226	-5 (2)	97	120	1.07 (3)	21	54
A	86	210	286	13 (2)	149	168	1.07 (2)	35	58
A	86	300	376	18 (4)	216 (3)	242	1.04 (5)	48	67
				4	93	106			

Solution A refers to the Ringer fluid of Barkan type described under 'Methods', and B the special fluid resembling closely the inorganic composition of frog's plasma. The bracketed figures refer to the number of experiments when more than one was performed. Sodium and potassium analyses were frequently carried out on the one muscle group.

The following points are clearly indicated:

(1) The fibres of the excised muscle have a practically perfect impermeability for sodium, when the external potassium is somewhat raised above the normal plasma value. Thus for the low-temperature experiments and with an external potassium concentration of 18 m.equiv./l. only 11 m.equiv. sodium enter per kg. over 24 hr. As the potassium is further increased the sodium is even better excluded so that a small loss is observed. This arises from a diminution of the sodium concentration in the interfibre fluid consequent on a fall of external sodium concentration from 104 (plasma value) to 86 m.equiv./l.

Similar improvement in the membrane permeability is shown at room temperature when the potassium concentration is raised.

(2) While sodium is strictly excluded, potassium enters in large amounts against a gradient, as the external potassium concentration is increased. Thus, with an external value of 150 m.equiv./l., the gain is 97 m.equiv./kg. or a total of 240 m.equiv./l. fibre water is reached.

Concerning the external potassium concentration required to ensure neither loss nor gain of the amount in the immersed muscle, Fenn & Cobb [1934] found with a Ringer solution containing $M/150$ phosphate and at a pH of 7.2, that a concentration of 5 m.equiv./l. (twice the plasma value) was necessary. Without the phosphate much higher values were

required, and this accords with our results since at room temperature (with 2 hr. immersions) we found it necessary to have an external potassium concentration of 29 m.equiv./l., though only about 12 were required over 24 hr. at low temperatures. The special action of phosphate here may be attributable to increased production of phosphate esters within the fibre with an effect to be described subsequently. (It may be mentioned that Mond & Amson [1928] found a maintenance concentration of potassium as low as 3.3 m.equiv./l. when they perfused the limbs of a frog with a fluid otherwise resembling Ringer's solution.)

(3) While the muscle manifests its characteristic differential permeability to potassium and not to sodium, it is freely permeable to chloride. Chloride enters in almost equivalent relation to the great increases of potassium. From the total average values in Table 1 for low-temperature immersions, there is a gain of 93 m.equiv. of potassium, 106 of chloride and 4 of sodium.

(4) From an examination of the cation and anion balance it appears that whereas the membrane is permeable to chloride it is impermeable to phosphocreatine, adenylypyrophosphoric acid, etc., or to the phosphate esters in general within the muscle. Thus, if we consider the mean values in the last paragraph and that a bicarbonate loss of about 2 m.equiv./kg. muscle from the interfibre fluid must at least occur in the change from the plasma value of 25 to the external fluid value of 12 m.equiv./l., we get a total cation gain of 97 and an anion gain of 104. These figures may differ only by the sampling error, or mean at most but a small interchange of chloride for anions previously indiffusible.

It appears, therefore, that with an improvement, or approach to normal, of the membrane permeability consequent on a raised external potassium concentration the excised sartorius exhibits a very definite and curious permeability. Sodium is strictly excluded and potassium let through freely. That the differentiation here is merely a question of the ion diameter in solution will appear in the discussion at the end. While permeable to potassium, the membrane is also freely permeable to chloride, but not to anions of the type of phosphate esters, colloidal anions or negatively charged protein.

For such a membrane theoretical equations for electrolyte equilibria, volume changes, and membrane potentials can be deduced as in the next section.

III. THEORETICAL EQUATIONS FOR THE ELECTROLYTE EQUILIBRIA ACROSS THE MEMBRANE

The concentrations on each side of the membrane may be represented as follows:

Concentrations in fibre water	Membrane	External concentrations
$\Sigma B/V$		Σb
k_1		k
$\Sigma d_1/V$		Σd
$\Sigma A/V$		Σa
$\Sigma C/V$		Σc
$\Sigma G/V$		Σg

$$\left. \begin{array}{l} \Sigma b \\ k \\ \Sigma d \\ \Sigma a \\ \Sigma c \\ \Sigma g \end{array} \right\} = c$$

The symbols have the following meaning, and refer to one type of substance only unless otherwise stated, the symbol Σ being used to represent the totality of such.

In 'fibre water'

- B, A Quantities of indiffusible non-colloidal cations and anions respectively and which may be considered as mM./l. of 'fibre water' in fresh excised muscle before immersion.
- C Quantity of colloidal anions, measured as for B and A .
- G Quantity of indiffusible non-electrolytes or uncharged molecules, measured like B and A .
- V Volume of 'fibre water' after immersion. For muscle before immersion, $V = 1$ l.
- k_1, d_1 Concentrations of potassium and diffusible anions as mM./l. 'fibre water' before or after immersion, according to the conditions specified.

For external solution

- b, a Concentrations of indiffusible cations and anions as mM./l.
- k, d Concentrations of potassium and diffusible anions as mM./l.
- g Concentration of uncharged molecules, as mM./l.
- c Total external concentration as mM./l.

Before considering the equations the following simplifications may be made. It may be taken that the diffusible anions are monovalent, being almost altogether chloride and bicarbonate for the conditions described. The phosphate concentration of frog's plasma or solutions resembling it is negligible, in a comparatively quantitative sense, and any further diffusible anions within the membrane will emerge and become negligibly small in concentration in the large volume of the external fluid.

these are of a kind that are realized within some hours. (This question is again considered in the discussion.)

With true equilibria as contrasted with a steady state involving free-energy transfers we may consider directly the application of the Donnan relation, from which

$$k_1 \times d_1 = k \times d \quad (5)$$

for any one diffusible anion, or in general

$$\Sigma k_1 \times d_1 = \Sigma k \times d. \quad (6)$$

(Such relations can be directly tested, and as will be seen for potassium and chloride the experimental results are most satisfactory.)

The object aimed at in the further development of these equilibrium conditions is to obtain equations relating k_1 , d_1 , V and π (the potential across the membrane) to the external electrolyte and total external concentrations, as well as to the 'constants' η and ϵ of the muscle fibre. With this object the following may be easily derived from equations (2), (4) and (5):

$$k_1 = \frac{1}{2}c - (\eta - \epsilon)/2V. \quad (7)$$

This equation is obtained from the addition of equations (2) and (4). Also

$$\Sigma d_1 = \frac{1}{2}c - (\eta + \epsilon)/2V, \quad (8)$$

from the subtraction of equations (2) and (4).

An equation giving Σd_1 in another and more useful form can be derived as follows. From equation (6)

$$\Sigma d_1 = \frac{\Sigma k \times d}{k_1},$$

and from equation (7) we have therefore

$$\Sigma d_1 = \frac{\Sigma 2kd}{c - (\eta - \epsilon)/V}. \quad (9)$$

An equation also giving V in terms of the external concentrations and η and ϵ may be derived as follows. If we square equations (2) and (4) and subtract we obtain

$$\Sigma 4k_1 d_1 = c^2 + (\eta/V)^2 - 2\eta c/V - (\epsilon/V)^2. \quad (10)$$

Since, from equation (6), $\Sigma kd = \Sigma k_1 d_1$ we may collect and arrange to

$$V^2 (c^2 - \Sigma 4kd) - 2\eta Vc + (\eta^2 - \epsilon^2) = 0, \quad (10a)$$

which gives V in the required form.

It may be reasonably assumed too that all univalent ions such as Na, K and Cl have the same activity coefficients, so that where such ions only are considered in solutions of the same or very similar ionic strengths we may in many cases deal with concentrations instead of activities.

For the immediate purpose, however, of stating the fundamental equations it will be simpler at the outset to consider that we are not dealing with the muscle fibre, but with a hypothetical fibre or chamber in which all concentrations are so low that activities do not appreciably differ from concentrations. Subsequently, the activity effects on the derived 'constants' will be considered, particularly with relation to change of external concentration.

It is also assumed that the membrane is practically inelastic and contributes no internal pressure, over the volume ranges studied, comparable to the total osmotic pressure. (Conversely, we can prove this point for the muscle membrane to a sufficient approximation when comparing later the volume changes with the change of composition within and without the muscle fibres.)

(1) *Osmotic equilibrium.* For this we have

$$\begin{aligned} \Sigma B/V + k_1 + \Sigma d_1 + \Sigma A/V + \Sigma C/V + \Sigma G/V \\ = \Sigma b + k + \Sigma d + \Sigma a + \Sigma g = c. \end{aligned} \quad (1)$$

$$\text{Writing} \quad \Sigma B + \Sigma A + \Sigma C + \Sigma G = \eta, \quad (1a)$$

$$\text{we have} \quad k_1 + \Sigma d_1 = c - \eta/V. \quad (2)$$

(2) *Electrical equilibrium.* Since the solutions within and without the membrane must be electrically neutral as a whole we have

$$k_1 + \Sigma mB/V = \Sigma d_1 + \Sigma pA/V + \Sigma qC/V, \quad (3)$$

where m , p and q are the valencies of the indiffusible base, indiffusible anions and colloidal anions within the fibre. (The total charged colloidal molecules may be taken as protein anions, considered as excess of negative over positive charges.)

The equation (3) may be written

$$k_1 - \Sigma d_1 = \epsilon/V, \quad (4)$$

$$\text{where} \quad \epsilon = \Sigma pA + \Sigma qC - \Sigma mB. \quad (4a)$$

(3) *The Donnan equilibrium.* The electrolyte balance in excised muscle relates, as we may assume, to true equilibria, involving no continual expenditure of energy. That this is so, or sufficiently so for our purpose, is shown if only by the fact that reduction to a temperature close to zero makes no appreciable difference in the relations to be described, provided

these are of a kind that are realized within some hours. (This question is again considered in the discussion.)

With true equilibria as contrasted with a steady state involving free-energy transfers we may consider directly the application of the Donnan relation, from which

$$k_1 \times d_1 = k \times d \quad (5)$$

for any one diffusible anion, or in general

$$\Sigma k_1 \times d_1 = \Sigma k \times d. \quad (6)$$

(Such relations can be directly tested, and as will be seen for potassium and chloride the experimental results are most satisfactory.)

The object aimed at in the further development of these equilibrium conditions is to obtain equations relating k_1 , d_1 , V and π (the potential across the membrane) to the external electrolyte and total external concentrations, as well as to the 'constants' η and ϵ of the muscle fibre. With this object the following may be easily derived from equations (2), (4) and (5):

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$$V^2 (c^2 - \Sigma 4k d) - 2\eta V c + (\eta^2 - \epsilon^2) = 0, \quad (10a)$$

which gives V in the required form.

Considering the potential differences π across the membrane we may write in the usual manner for a Donnan relation

$$\pi = \frac{RT}{F} \ln k_1/k,$$

which from equation (7)

$$= \frac{RT}{F} \ln \frac{c - (\eta - \epsilon)/V}{2k},$$

$$\text{or} \quad = 58 \log \frac{c - (\eta - \epsilon)/V}{2k}, \quad (11)$$

as millivolts at room temperature (18° C.). As will be shown subsequently the values of η and ϵ are by accident practically identical in value for the sartorius muscle of the frog, so that equations (7), (9), (10a) and (11) would simplify to

$$k_1 = \frac{1}{2}c, \quad (12)$$

$$\Sigma d_1 = \Sigma 2kd/c, \quad (13)$$

$$V = \frac{2\eta c}{c^2 - \Sigma 4kd}, \quad (13a)$$

$$\pi = 58 \log c/2k. \quad (13b)$$

It has been shown therefore that the potassium concentration within the hypothetical fibre (or fibre with concentrations so low that activities did not differ appreciably from concentrations), the 'fibre water' volume, and the potential across the membrane can be expressed in terms of the external concentrations and certain constants η and ϵ related to the indiffusible substances within.

We may now inquire how our working equations (7), (9), (10a) and (11) (or their simplifications (12), (13), (13a) and (13b)) may need to be altered when considering the actual muscle fibre. These equations have been derived from the three fundamental equations (2), (4) and (6). Taking equation (2), the effect on the activity of the solvent is now not given by the analytical concentrations as stated, but by these multiplied by some factor (not identical with the activity coefficient, as considered below) which we may suppose to be the same for the univalent ions k_1 and Σd_1 , and also for c which may be taken as made up almost entirely of univalent ions. Inserting the analytical concentrations of k_1 and Σd_1 and c therefore in equation (2) will not give the real value of η but an *apparent* value related thereto. This is of no consequence for our purpose provided the apparent η value remains constant with changes in the external potassium concentration. This question may then be considered.

Constancy of apparent η values with changing external potassium. The increased potassium concentration will bring about two kinds of change that may be significant, an increase of ionic strength (with its effect on activities) when the total external concentration is raised

and a change of pH. The change in pH need not be considered since this cannot affect the stoichiometric or analytical value of η . With changing ionic strength, however, the *apparent* value of η , as determined from equation (2), will alter on inserting the *analytical* values of k_1 , Σd_1 and c . The case where the external concentration is maintained constant and potassium substituted for sodium we need not consider, the change being significant only when c is raised, as it is in the experimental investigation from 0.2 to 0.6 *M* when the internal concentration in the fibre water will vary to the same extent.

Considering then the equation (2)

$$k_1 + \Sigma d_1 = c - \eta/V,$$

the underlying equivalence here is the activity of the solvent on each side of the membrane, and for very dilute solutions we may suppose each molecular species affecting this in proportion to its concentration. With more concentrated solutions it is necessary to consider activities. It would appear, however, that solute and solvent activities cannot be theoretically related at present, for although the equation of Lewis & Linhart [1919] links activities with freezing-points, this is done only by the introduction of arbitrary or experimental constants which vary somewhat for each substance. We may, however, approach the question somewhat indirectly as follows. If η were made up entirely of monovalent electrolytes the question would not arise, since over the concentrations studied all monovalent electrolytes would not differ significantly for our purpose in their effect on the freezing-point. If we suppose it to be made up of divalent anions, and this, from a consideration of the muscle composition, will introduce a greater source of error than the actual, then we may consider the following figures. From the data in the *International Critical Tables*, 0.079 *M* K_2SO_4 should be in osmotic equilibrium across a semipermeable membrane with 0.1 *M* NaCl, both solutions giving the same freezing-point. If to the NaCl solution KCl be added to make a 0.2 *M* KCl, then to the other KCl must be added to the extent of 0.204 *M* (determined by freezing-point data with ordinary Beckmann procedure). Regarding the sulphate ion as η we could insert these values in an equation of the same form as (2) (where $V=1$, the equation representing merely the condition of osmotic equilibrium); c being 200 m.equiv./l. in one case and 600 in the other, we would obtain for η 42 and 38 respectively. This would signify a fall of 9%. Seeing that the actual value of η for the sartorius muscle is about 106 and that it enters the equation for the calculation of the internal potassium concentration, this value changing to about 300 m.equiv./l., the error involved in taking apparent η values as constant would be only 1 or 2%. *As the case discussed overstates the probable change we may safely regard η as theoretically constant in using the equations for calculating k_1 , etc., for the muscle fibre.*

The fact that 0.079 *M* K_2SO_4 is in osmotic equilibrium with 0.1 *M* NaCl should recall a certain caution in the investigation of balances based on stoichiometric concentrations of ions in tissues and plasma, since here 237 actual mM./l. are in equilibrium with 200. Thus we find Hill & Kupalow [1930] and others seeking exact balances of such quantities across the muscle membrane.

The constancy of ϵ values. Considering equation (3), which expresses the electroneutrality of the solution as a whole, the values of ϵ (equation (4)) will give actual or analytical concentrations. When in the value of ϵ (i.e. of $k_1 - \Sigma d_1$) k_1 and Σd_1 increase, the total change is zero unless more negative or basic charges appear from dissociating weak acids or bases within the muscle. Since the hydrogen ion will fall inside as the external potassium increases (because of the falling ratio of potassium across the membrane) an increase will occur in the total balance of anion charges from dissociating molecules within. Over the experimental range examined the pH in the fibre may be expected to change from 6.4 to 7.2. Negative charges on indiffusible anions will increase from the further dissociation of adenylypyrophosphoric acid, hexosemonophosphoric acid and the muscle proteins, and positive charges will disappear with diminished dissociation of the second equilibrium constant of carnosine. We can make a reasonable estimate of the sum of these changes, as given in Table 3. (Data for this table have been taken in part from Table 2, which gives

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TABLE 2. The lactate, bicarbonate, phosphate and sulphate concentrations in muscle have been calculated assuming similar plasma/muscle ratios as for chloride, this being further considered in the discussion at end. The calculations of the concentrations in the 'fibre water' have been made in accordance with the previous paper and referred to under 'Methods'. Data marked:

(F)	represents Fenn [1936]
(E)	„ Eggleton [1935]
(B)	„ Barkan <i>et al.</i> [1921]
(D)	„ Duff [1932]
(S)	„ Schulz <i>et al.</i> [1925]
(C)	„ Conway <i>et al.</i> [present paper, 1934 and 1935].

The data for potassium, sodium and chloride concentrations in muscle have been obtained on 89, 34 and 21 experiments respectively (usually four muscles for each). The plus and minus figures refer to the standard deviation of the means. For the mean sodium concentration two high values were discarded.

Muscle mM./kg.		Plasma mM./kg.	Allowance for inter- fibre space mM./kg.	'Fibre water' mM./l.
K	84.6 \pm 1.0 (C)	2.5 (F)	0.3	126
Na	23.9 \pm 0.6 (C)	103.8 (F)	13.5	15.5
Ca	2.5	2.0 (F)	0.3	3.3
Mg	11.3 \pm 0.2 (C)	1.2 (C)	0.2	16.7
Carnosine	11.0 (E)	—	—	16.4
Hexose-mono- phosphoric acid	1.9 (E)	—	—	2.8
Phosphagen	25.8 (E)	—	—	38.5
A.T.P.	3.2 (F)	—	—	4.8
Protein	1.6	0.6	—	2.3
Chloride	10.5 \pm 0.4 (C)	74.3 (F)	9.7	1.2
Lactate	0.5	3.3 (F)	0.4	—
Bicarbonate	3.6	25.4 (F)	3.3	0.4
Phosphate	0.4	3.1 (F)	0.4	—
Sulphate	0.3	1.9 (C)	0.3	—
Glucose	0.5	3.9 (B)	0.5	—
Water	800/g./kg.	954/g. (S)		

a summary of the composition of frog's muscle and plasma; details of the calculations are given with Table 3.) Over the external range of 12–210 m.equiv. potassium/l. the total change of ϵ is about 22 m.equiv./l. of fibre water, the greater part of this change being due to protein. On the mean value of 124 for ϵ this means an increase of 18%, but in computing k_1 from equation (7) it involves an error of only 2–4% when there is no volume change. When there is a volume change, ϵ will be proportionally decreased so that at no time should the error exceed about 5%.

The effect so calculated should be apparently somewhat less, since the activity change of the hydrogen ion, owing to the rising ionic strength, will be smaller than the change in actual concentration; but this in turn will tend to be counterbalanced by the fall in pK' values resulting in an increase of anions (or fall of cations) at a given fixed pH value. These two effects not only run counter to each other, but are each of much smaller magnitude than the large change in hydrogen ion considered above, and may therefore be neglected.

Since the membrane potential is calculated from k_1 (as derived from equation (7)), similar considerations of accuracy apply, as they will also for the calculation of V (vide also § IV (f) below for activities and the Donnan equilibria). The net result is that, for the determination of these variables from the foregoing equations we may consider both η and ϵ as theoretically constant for the muscle fibre over the range considered.

TABLE 3. The pK value of adenylypyrophosphoric acid, representing the fourth and last acidic equilibrium constant was taken from Lohmann's diagram [1932] of the electro-metric titration, in which half the fourth equivalent of the base added gave a pH of 6.2. From the ionic strength of the solution an estimate of 6.4 may be made of the true equilibrium constant. The pK value of hexose-monophosphoric acid is taken from Irving & Fischer's figures [1927] using the difference between their estimate of this value and that for phosphoric acid (second equilibrium constant) to subtract from Cohen's figure [1927] of 7.16 for the latter. The pK value for the second equilibrium constant of carnosine was taken directly from Deutsch & Eggleton's determination [1938] since the ionic strength of the solution was comparatively low, and considering also that a change of the order of 0.2 pH would amount to only 0.8 m.equiv./l. in the final computation.

We have considered the increase in the negative charges on the muscle proteins as analogous to those on serum globulin and serum albumin. From the data of Peters & Van Slyke [1932] and considering that 1 l. of 'fibre water' will be associated with 230 g. protein, the m.equiv. extra base bound from pH 6.4 to pH 7.2 may be expected as 11.13 or a mean of 12 m.equiv./l. of 'fibre water'.

The calculations have been made for room temperature, but the total effect is probably little different at 2-3° C.

Substance	mM./l. of 'fibre water'	pK	Total increase of anion and loss of cation m.equiv. from pH 6.4 to 7.2
Protein	2.3	—	12.0
Carnosine	16.4	6.8	7.0
Adenyl-pyrophosphoric acid	4.8	6.4	1.7
Hexose monophosphoric acid	2.8	6.5	0.8
Phosphocreatine	38.5	4.5	0.4
			<hr/> 21.9

IV. EXPERIMENTAL RESULTS AND THE THEORETICAL EQUATIONS

(a) The values of η and ϵ

These are considered first, since on the absolute or relative constancy of their mean experimental values turns the worth of the equations for determining k , V , etc., under changing conditions. We shall examine η and ϵ in vivo prior to their calculation for muscle after prolonged immersion in the cold and in solutions containing widely varying potassium concentrations. Concentrations in the 'fibre water' are calculated as given under Methods, the question of the 'osmotically active' water being dealt with below.

η and ϵ for muscle in vivo. From Table 2 the mean values of potassium and of chloride concentrations in the fibre water are 126 and 1.2 respectively. Assuming that the real bicarbonate behaves similarly to chloride its concentration is 0.4 and the total value of Σd_1 is approximately 2, so that $\epsilon (=k_1 - \Sigma d_1)$ is 124. Similarly, η (or $c - k_1 - \Sigma d_1$) is 105, since c is here the plasma concentration, 222/0.954 or 233, from Table 2. This value of 233 for the plasma omits the freely diffusible non-electrolytes

such as urea, creatine, etc., and also amino acids. The figure of Hill & Kupalow [1930] for the total plasma concentration is the equivalent of 0.75 % NaCl or 256 mM./l.

The values of η and ϵ so given are general mean values, but some variation occurs from one consignment to another. For the group of immersed muscles considered below, the potassium content of a certain number of companion muscles was 121 rather than 126, so that η and ϵ appear as 110 and 119.

η and ϵ in immersed sartorii with wide range of external potassium concentration. The sartorii were immersed for 24-hr. at 2–3° C. in Barkan fluid containing 3–300 m.equiv. potassium, and prepared as discussed under 'Methods'. For each immersion four muscles from four frogs were used, and for many groups the companion tissues were also analysed to obtain the original potassium content. These experiments were designed to show mean changes of V (fibre water, the original V value being 1.0) from 1.17 to 1.14 over the potassium range, that is, practically constant throughout, but slightly raised over the normal. (The manner of doing this is discussed in the next section.)

The results are shown in Table 4. Large changes in muscle potassium occur with no corresponding increase in V which deviates from the theoretical by at most 7.7 %. It will be seen that with marked increase of k , η and ϵ do not vary significantly, with the exception of a fall in ϵ at the extreme range of 300 m.equiv. potassium/l.

TABLE 4

External concentrations			Concentrations in the 'fibre water'			V 'fibre water' after/ before	η	ϵ
Potas- sium k	Chloride	Total c	Potassium k	Chloride	Bicar- bonate (calc.)			
3	79	186	91 (3)	7.2 (2)	1.1	1.14	99	95
6	82	192	92 (3)	7.2 (3)	1.1	1.14	104	96
12	88	204	101 (3)	9.9 (2)	1.3	1.26	116	113
18	94	216	107 (3)	16.1	2.0	1.23	112	109
30	106	240	120 (4)	24.9 (3)	2.8	1.17	108	108
60	136	300	142	60.6	5.2	1.12	103	85
90	166	360	184	86.0	6.2	1.13	95	104
120	196	420	212	114.2	6.9	1.12	98	103
150	226	480	240 (2)	143.1	7.5	1.09	98	98
210	286	600	282 (4)	186.7 (2)	7.7	1.06	131	93
300	376	780	353 (3)	308 (2)	9.9	1.07	117	38

The bracketed figures refer to the number of experiments when more than one was performed. All concentrations given as mM./l.

Variations of η and ϵ from one experiment to another in Table 4 are such as may be expected from similar variations in the original muscle.

The median η and ϵ values over the significant range of 12–210 m.equiv./l. of outside potassium are 105.5 and 103.5. Comparing these with 110 and 119, as found for fresh muscle, η is little altered. As shown in a previous section, we must consider, however, a small mean increase of sodium amounting to about 7 m.equiv./l. of fibre water, leaving 11 m.equiv. altogether to be accounted therefore as loss of some previously indiffusible material. It is true that half of this could be attributed to a salt effect on the apparent value as considered in the previous theoretical section, but since the example chosen there rather exaggerates the salt effect this point is of little significance.

From the nature of ϵ amounting to the difference between the total charges on the indiffusible anion and cation concentrations, the gain of sodium and the loss of the indiffusible substance—if this be an electrolyte—will *both* diminish the result. From the resultant mean of 103.5 with loss of a monovalent electrolyte and corresponding gain of 7.5 m.equiv. sodium, the original figure should be 118.5, or 127 if the loss be attributable to phosphocreatine.

The main results may be given as:

(1) η and ϵ are even more constant for the immersed muscle than could be expected theoretically, and this may be attributed to slight mean gains of sodium and losses of indiffusible anions.

(2) The mean values of η and ϵ differ by only 2 m.equiv./l. and for practical purposes are therefore identical.

This agreement of η and ϵ is accidental and may not apply even to other skeletal muscles of the frog, but it makes possible a much simpler theoretical treatment of the volume changes.

Direct or analytical computation of η . Calculations of η from equation (2) are indirect and give also apparent rather than real values, as exemplified in a previous section. From Table 2 we may compute η directly. It may be summed as follows:

Phosphocreatine	38.5
Magnesium	16.7
Carnosine	16.4
Sodium	15.5
Adenylpyrophosphoric acid	4.8
Calcium	3.3
Hexose monophosphoric acid	2.8
Protein	2.3
	<hr/> 100.3

This is very close to the general mean value of 105 calculated indirectly, but at the same time we must consider that most if not all the calcium will be probably combined with colloidal molecules and exert no appre-

cial influence, also that magnesium is almost all organically combined [Cruess-Callaghan, 1935], but this combination may occur with small molecules (for magnesium is known to combine with such) and so reach possibly the full value given. It may be noted too that we should expect the directly summed value to exceed somewhat the indirect owing to the comparatively smaller effect of divalent or higher valency ions, when expressed stoichiometrically on the water activity.

Direct computations of ϵ are comparatively without value until the charges on the protein molecules are exactly known.

(b) *Potassium accumulation with wide volume changes but constant potassium concentration in muscle*

The solution of equation (10), or

may be given as
$$V^2(c^2 - \Sigma 4kd) - 2V\eta c + (\eta^2 - \epsilon^2) = 0,$$

$$\begin{aligned} V &= \frac{\eta c}{c^2 - \Sigma 4kd} + \frac{\eta c}{c^2 - \Sigma 4kd} \sqrt{\left\{1 - \frac{(\eta^2 - \epsilon^2)(c^2 - \Sigma 4kd)}{\eta^2 c^2}\right\}} \\ &= \frac{\eta c}{c^2 - \Sigma 4kd} + \frac{\eta c}{c^2 - \Sigma 4kd} \sqrt{\left\{1 - \left(1 - \frac{\epsilon^2}{\eta^2}\right)\left(1 - \frac{\Sigma 4kd}{c^2}\right)\right\}}, \end{aligned} \quad (14)$$

in order to facilitate the calculation of the error involved in simplifying to equation (13a), or

$$V = \frac{2\eta c}{c^2 - \Sigma 4kd}.$$

If in the external solution univalent electrolytes are almost entirely present, $\Sigma 2d$ cannot be greater than c , and $2k$ also cannot be greater than c , so that the value of $(1 - \Sigma 4kd/c^2)$ ranges between 1.0 and zero. Taking the maximum value of 1.0 it will appear then from equation (14) that where η is 20% greater than ϵ , the greatest error involved in the simplification to equation (13a) is 8.5%, but will be less than this when potassium chloride is present in the external solution in amounts ranging from 10 m.equiv./l. upwards.

For our present purpose, however, practically no error is involved, since the experimental investigation has shown η and ϵ to differ in their mean values by only 2%. Consequently we may regard the simplified equation as accurately expressing the theoretical relations.

For the conditions required for obtaining the widest volume changes with constant potassium concentration the following equations ((7) and (12) with simplifications) may now be considered:

$$\begin{aligned} k_1 &= \frac{1}{2}c - (\eta - \epsilon)/2V \\ &= \frac{1}{2}c, \end{aligned} \quad (15)$$

and
$$V = \frac{2\eta c}{c^2 - \Sigma 4kd} = \frac{211}{c - 2k}, \quad (16)$$

since 2η for the immersed muscle has been shown to be 211 , and $\Sigma 2d$ is the same as c when the external solution is entirely or almost entirely composed of univalent electrolytes.

For a constant potassium concentration therefore it is only necessary from equation (15) that c be constant, and that with change of k externally, potassium be substituted equivalently for sodium.

With c constant the widest increases of V may be had by increasing k progressively, as in equation (16).

Table 5 shows the results obtained. The changes in muscle weight for the individual experiments are given as well as the average weights and values of V . Up to 100 % theoretical increase in the mean change

TABLE 5

k (potassium conc. in external fluid) m.equiv./l.	Change of muscle wt. g./100 g.	Mean change in wt. (median values)		Mean value of 'fibre water'		Potassium conc. in 'fibre water' m.equiv./l.
		Exp.	Theor.	Exp.	Theor.	
10	6.9; 0.0; -2.7	0.0	-3.3	1.00	0.95	119 (1)
20	9.6; 4.8; 5.2	5.2	2.7	1.08	1.04	—
30	6.7; 22.8; 9.2; 10.3; 11.0; 6.2	9.7	10.0	1.14	1.15	120 (4)
40	31.0; 20.2; 11.1; 20.6	20.4	19.4	1.30	1.29	119 (1)
50	32.0; 26.6; 34.0; 28.6	30.3	30.8	1.45	1.46	—
60	56.8; 35.3; 40.2; 41.2; 44.1	41.2	46.8	1.62	1.70	—
70	72.1; 65.0; 64.0; 51.5; 47.1	64.0	68.0	1.95	2.03	—
80	95.0; 89.0; 75.2	75.2	99.0	2.12	2.48	117 (1)
90	104.3; 103.0	103.6	151.0	2.55	3.25	—
100	115.1; 89.0	102.1	247.0	2.52	4.69	—

The external fluid was of the Ringer-Barkan type described in 'Methods', the total concentration being 243 mM./l. and maintained constant throughout with varying proportions of KCl and NaCl. The total diffusible anion Σd had a constant value of 120 mM./l. The theoretical value of V was reckoned from the equation

$$V = \frac{2\eta}{c - \Sigma Akd/c} = \frac{211}{243 - 1.98k}$$

The theoretical value for the percentage increase in muscle weight was calculated from the formula $67(V-1)$, V being obtained from the above equation.

of V the experimental mean agrees very well, differing throughout this range by 5 % at most. Beyond this 100 % increase of V , or beyond an external k value of 70 m.equiv./l., V rapidly reaches a maximum and consequently falls markedly below the theoretical figure.

Table 5 shows also how the potassium concentration in the muscle fibre does not alter with wide changes in V and consequently considerable potassium accumulation. Thus at an external k of 80, the potassium

concentration in the fibres is unchanged but they have accumulated 78 m.equiv./kg. muscle.

In the calculation of the increase of fibre water, it has been assumed that the total change in the muscle weight has been due to this increase, while the water in the interfibre spaces remains constant. It may be supposed, however, that this interspace water is progressively squeezed out as the muscle swells. It amounts to 9–13 ml./100 g. in fresh muscle, and as it is pressed out in part with rising volume, the real change in the fibre water should approach even more closely to the theoretical.

Increase of muscle volume in isotonic potassium chloride solutions was observed by Overton [1904] and considered by Höber [1922] without its true nature being understood.

It was also shown by Siebeck [1913] and Meigs, as quoted by Höber [1922], that the water uptake was reversible when up to 40 % increase of the 'fibre water' (as we would describe it) occurred. For the sartorius in the cold the increase up to 100 % goes in accordance with the theoretical equation above, but whether it is then reversible we have not examined, but would not be surprised if it were not fully reversible, since it takes 24 hr. to reach equilibrium and hence presumably another 24 hr. to reverse, in which latter period the muscle may be expected to show some deterioration.

Equilibration times. So far we have assumed that 24 hr. immersion, without stirring, at 2–3° C., or 2 hr. immersion at room temperature with bubbling, is sufficient for equilibrium, provided in the latter case there is no marked increase in volume but only in concentration.

As evidence for this, Fig. 1 shows the volume changes with time of immersion at 2–3° C., the conditions being the same as for Table 5. For each curve a single group of four immersed muscles was used, being dried quickly, weighed and re-immersed at the stated intervals. It will be seen that the curves for different potassium concentrations outside (but constant total external concentration of 243 m.equiv./l.) rise rather slowly to their maximum, reaching this in about 16 hr., remaining then practically steady until 24 hr. and then declining slowly over several days. In the period of 16–24 hr. the volume change is probably close to a true equilibrium and corresponds as we have seen to the theoretical requirements.

The slow rise with changing volume is not at all suitable for room-temperature observations, since the conditions of the muscle or muscle membrane deteriorate after some hours and the true maximum is never reached, especially when the volume change is great. This is shown in Fig. 2.

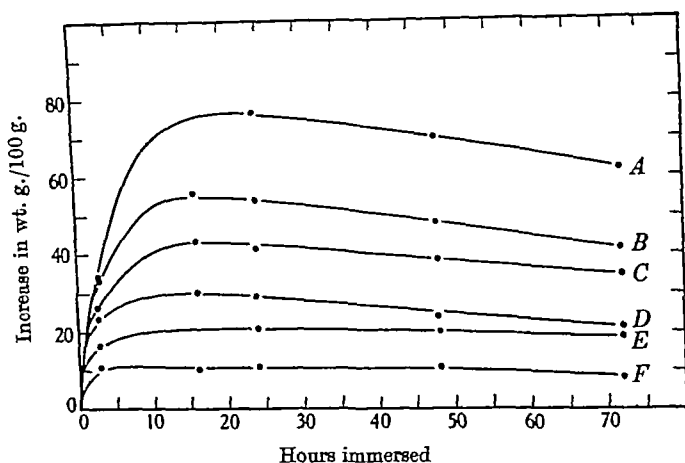


Fig. 1. Increase in weight of sartorii (four for each experiment) immersed at 2-3° C. in solution of Ringer-Barkan type, of constant total concentration (243 mM./l.) and varying potassium content. $pH=7.3$.

A is curve for 80 m.equiv. potassium/l.
 B is curve for 70 m.equiv. potassium/l.
 C is curve for 60 m.equiv. potassium/l.

D is curve for 50 m.equiv. potassium/l.
 E is curve for 40 m.equiv. potassium/l.
 F is curve for 30 m.equiv. potassium/l.

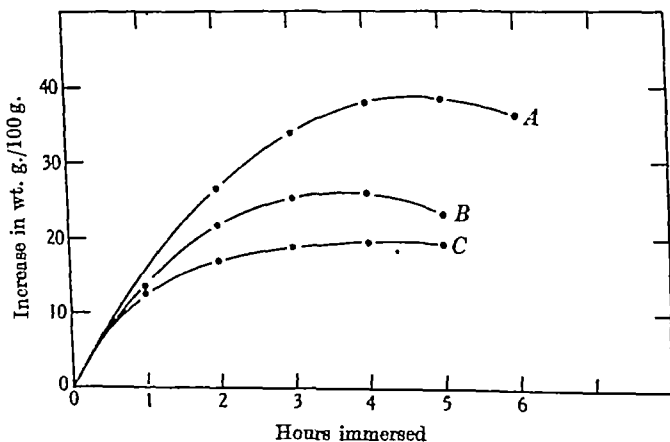


Fig. 2. Increase in weight of sartorii at room temperature. Conditions otherwise same as for Fig. 1.

A is curve for 80 m.equiv. potassium/l.
 B is curve for 70 m.equiv. potassium/l.
 C is curve for 60 m.equiv. potassium/l.

The curves tend to a maximum at the fourth or fifth hour, but it is only about half or less of that reached with immersions in the cold.

While this is so with marked volume increases, there is otherwise a potassium concentration equilibrium reached in 2 hr. Observations of potassium concentrations after 2 hr. immersion with stirring at room temperature do not differ appreciably from observations at 4 hr. periods, nor for 24 hr. immersions in the cold.

(c) *Potassium accumulation at constant volume with marked changes of potassium concentration, within the muscle fibres*

The conditions for maintaining a constant volume in spite of large potassium increases in the external solution may be easily deduced from equations (15) and (16).

We shall suppose that the external solution is made up of monovalent electrolytes with diffusible anions so that $\Sigma 2d$ is equal to c . The Barkan solution used corresponds approximately to such conditions, there being only a small amount of calcium, phosphate and glucose.

We have then from equation (16) that

$$V = \frac{2\eta}{c - 2k},$$

but since c is made up almost altogether of sodium and potassium chloride and bicarbonate we may write

$$\begin{aligned} V &= \frac{2\eta}{(2[\text{Na}] + 2k) - 2k} \\ &= \frac{\eta}{[\text{Na}]} \\ &= \frac{105.5}{[\text{Na}]} \end{aligned} \quad (17)$$

If V is to remain constant throughout therefore, it is only necessary to maintain the external sodium-ion concentration constant. The sodium-ion concentration for any required V follows from

$$[\text{Na}] = \eta/V. \quad (17a)$$

While this gives the solution of the simplest case, equations for more complex conditions in the external solution may also be deduced.

We selected a constant V of approximately 1.17 corresponding to a muscle weight increase of 10 %, since we had a number of observations already collected with external potassium of 30 m.equiv./l. and mean weight increase of 10 %. The immersion solution contained per litre 50 ml. of 4.47 % KCl (30 m.equiv.), 363 ml. of 1.17 % NaCl (72.6 m.equiv.), and the remainder as described for the Barkan solution under 'Methods'. Throughout the series these residual materials plus the sodium chloride

were maintained constant, the volume per litre from the stock KCl solution being varied.

Apart from the added KCl the solution had the following composition (mM./l.):

Na	85.9
P	0.8
Ca	1.8
HCO ₃	11.9
Cl	76.2
Glucose	3.9
	<hr/> 180.5

Diffusible anions (Σd) = 88.9.

With 12 or 300 m.equiv. KCl in addition we would have c 204 or 780. From the simplified equation (17) and a sodium value of 85.9 we should expect a constant V of 1.23 or a constant weight increase on immersion of $23 \times 0.67\%$, or 15% . The presence of the glucose and calcium makes some difference, however, and to get the exact change from an external k value ranging from 12 to 300 we may use the equation (16)

$$V = \frac{211}{c - \Sigma kd/c},$$

where c is 204 and 780 and d is 101 and 389. V is therefore 1.17 and 1.15 or the percentage increase in weight is 11 to 10%.

The upper part of Table 6 shows the results of a number of experiments arranged on the above considerations. It will be seen that the volume change, considering the great accumulation of potassium, goes rather well with the theoretical, the maximum difference from this amounting to only 7%.

The table shows also how well the simple equation (15)

$$k_1 = \frac{1}{2}c$$

predicts the potassium concentration within the fibre, both for the 24 hr. immersion in the cold or for the 2 hr. room-temperature immersions. (From subsequent considerations the figures in the table below an external potassium value of 10 m.equiv./l. probably do not relate to true equilibria.)

For the 2 hr. immersion group no exact plan was followed except a maintenance of the volume either below that of the fresh muscle or very little above it. In the last five groups of experiments in Table 6 glucose was used for this purpose ranging from 1 to 5%, and it will be seen that it acts efficiently, the total c behaving as theoretically expected.

TABLE 6

Concentrations			Vol. of fibre water (mean values)		Potassium conc. in fibre water m.equiv./l.		Potassium accumulated m.equiv./kg. original muscle
Potassium in external fluid	Diffusible anion in external fluid	Total external	Exp.	Theor.	Exp.	Theor.	
24 hr. immersions without stirring at 2-3° C.							
3	91	186	1.14	1.17	91 (3)	93	-6.8
6	95	192	1.14	1.17	92 (3)	96	-2.6
12	101	204	1.26	1.17	101 (3)	102	18.6
18	107	216	1.23	1.17	107 (3)	108	13.4
30	119	240	1.17	1.17	120 (4)	120	18.2
60	149	300	1.12	1.16	142	150	35.7
90	179	360	1.13	1.16	184	180	72.4
120	209	420	1.12	1.16	212	210	98.4
150	239	480	1.09	1.16	240 (2)	240	110.8
210	299	600	1.06	1.15	282 (4)	300	141.0
300	389	780	1.07	1.15	353 (3)	390	233.4
2 hr. immersions with stirring at room temperature							
2.5	108	240	0.94	0.90	114 (3)	120	-10.9
4.0	116	240	0.99	0.92	120 (3)	120	-13.1
20.0	116	240	0.98	1.05	119 (5)	120	-1.2
27.0	116	240	1.15	1.12	95 (2)	120	-3.7
33.5	116	240	1.15	1.20	122 (5)	120	11.9
81	149	350	0.94	0.99	167 (5)	175	31.3
103	125	367	0.81	0.93	198 (5)	183	32.8
104	108	384	0.75	0.79	204 (2)	192	33.8
103	125	534	0.55	0.48	270 (3)	267	15.4
4 hr. immersion with stirring at room temperature							
104	108	384	0.76	0.79	193 (1)	192	31.2

Bracketed figures refer to number of experiments, when more than one was performed. Theoretical values of V calculated from equation (12) in text. In the last five experiments listed the external fluid contained 1, 2, 3, 5 and 3% glucose respectively. For the 24 hr. immersions the fluid was the same as for the experiments in Table 4 and described in § IV (c) of text. For the 2 hr. immersions at room temperature the solution was of the Ringer-Barkan type with varying KCl, NaCl and glucose additions. In the first experiment of this group the special fluid described under 'Methods' was used.

In Fig. 3 the results of the low-temperature immersions are presented in a different way. *The dots give the total concentrations per kg. muscle after immersion (concentrations being referred to the weight of the fresh muscle), and the line gives the theoretical mean line from the equation:*

$$C_m = (0.67 + a) \times k_1 + 0.13k,$$

which from equation (15)

$$= (0.67 + a) \times \frac{1}{2}c + 0.13k. \quad (18)$$

C_m represents the milliequivalents of potassium in the muscle after immersion (but referred to 1 kg. original muscle), and a the increase in l./kg. after immersion. This assumes that the increase in weight is an increase in 'fibre water', which from the results concerning volume

changes may be taken as sufficiently exact. Equation (18) is derived from the equation in a previous paper (and referred to here under 'Methods') for calculating concentrations in the 'fibre water'. (If concentrations

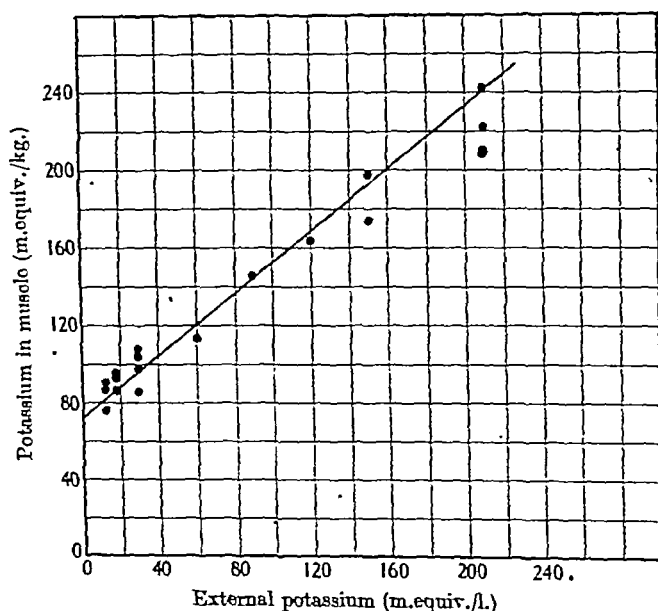


Fig. 3. Total potassium concentration in sartorii (m.equiv./kg. original weight) against potassium concentration in external fluid containing constant sodium concentration (85.9 m.equiv./l.). Immersions for 24 hr. at 2-3° C., conditions same as for Table 4 and described in text. The line gives the theoretical relation from equation (18) in text. The dots are for single experiments (four muscles each).

were referred to 1 kg. muscle after immersion Fig. 3 would be very similar, but the dots and line would run parallel at about 10% lower level.)

(d) *Potassium accumulation and the proportion of 'osmotically active' water in muscle*

We have considered so far that the proportion of the 'free' water in muscle contained within osmotically distensible membranes was given by the total 'free' water (as determined by vapour-pressure determinations or urea equilibria) minus the interspace water, and this we have termed the 'fibre' water. This may now be justified with respect to potassium accumulation, in a manner which brings independent evidence for the 'free water' in the fibres. It may be noted that the early work on this subject has been reviewed by Hill [1930] who repeated and extended the experiments of Overton [1902], criticizing the latter's

finding of a high proportion of 'bound' water. Hill obtained in his treatment a figure of 0.48 for the 'osmotically active' fraction in gastrocnemii immersed over many hours, and explained the difference between this and 0.64, or his estimate of the expected value, by assuming a breakdown in permeability in a proportion of the fibres. Our results are in agreement with Hill's view that there is no appreciable amount of 'bound' water in muscle, but it will be seen that any exact demonstration of the osmotically active water is considerably more complicated than was previously believed, since the membrane is permeable to both potassium and chloride ions. If immersed in ordinary Ringer fluid considerable losses of potassium will also occur (as shown in a later section), and this is not due to the membranes becoming permeable in a number of the fibres but to a slow loss from each. It is prevented by a certain maintenance concentration of the external potassium, but if any marked change of muscle volume is then brought about, new equilibria are formed and there may be large interchanges of potassium and chloride. Determinations of the 'osmotically active' fraction such as those of Fenn [1936] are also vitiated by the assumption that the chloride content gives a true measure of the interspace volume, which introduces a considerable error for excised and immersed muscle.

From the results discussed here the matter can now be resolved in more ways than one, but the following is perhaps the simplest and the most significant for our purpose. We know that with raised potassium concentration and over 24 hr. in the cold, sodium does not increase in the fibres but potassium may do so to a very large extent, yet the muscle volume can be maintained constant by simple adjustment of the external composition. The entering potassium must have passed across the muscle membrane and the problem resolves into demonstrating the amount of water in which it is dissolved. From equation (7) at constant normal volume we have

$$2k_0/\alpha = c - (\eta - \epsilon), \quad (19)$$

in which k_0 is somewhat different from the k_1 of equation (7), being the concentration of potassium *per kg. of muscle fibres*, or m.equiv./kg. of original muscle minus 0.13 time the external K as *effective* interspace allowance for electrolytes [Boyle *et al.* 1941]. α is the water inside the membranes (given as a fraction of the whole muscle) in which the potassium is dissolved. Since $(\eta - \epsilon)$ refers to the indiffusible material and remains practically constant over wide changes of potassium, the slope of the curve of $2k_0$ against c gives the value of α . The experiments described for varying potassium concentrations but constant volume in

the previous section, were designed to secure a small constant increase over the normal volume. This will have no appreciable effect on the absolute value of $(\eta - \epsilon)$ which is very small compared with c (no matter what value may be assigned to α within wide possible limits). We may write then

$$2k_0/(\alpha + \beta) = c - (\eta - \epsilon), \quad (20)$$

where β is the fractional increase in weight of the muscle after immersion, and being small may be attributed entirely to an increase of 'fibre water'. Hence

$$2k_0 - \beta c = \alpha c - a, \quad (21)$$

a being relatively small and constant. The slope of $2k_0 - \beta c$ against c will then give the value of α . Fig. 4 illustrates the data of mean values corresponding to those given in Table 4. The slope of the line is 0.675,

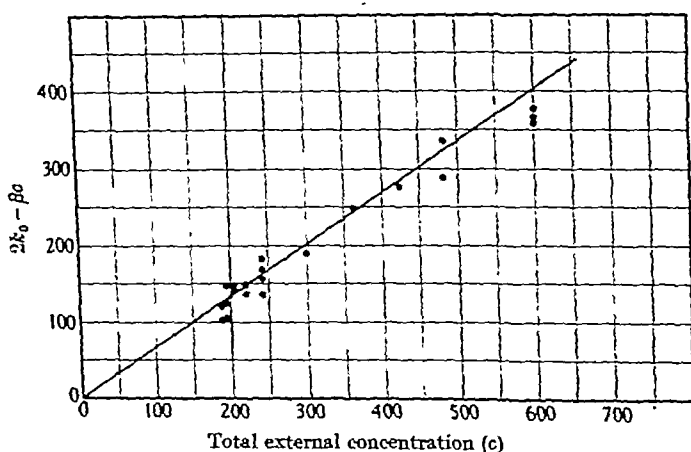


Fig. 4. The slope of the line (0.675) gives the water as a fraction of the whole muscle in which the normal potassium, or accumulated potassium at practically constant volume is dissolved. k_0 or the concentration of potassium in the fibres per kg. original muscle is obtained from Table 4 as $k \times V$. β or the fractional increase of muscle weight after immersion may also be obtained from Table 4 by subtracting 1.00 from Γ and multiplying by 0.67.

which is practically identical with the expected value of 0.67. The water in which potassium accumulated at constant volume, or the original potassium is dissolved, is therefore scarcely to be differentiated from the normal total muscle water (0.80) minus the interspace water (taken as 0.13, from previous paper).

(e) *Potassium accumulation and the diffusible anions*

As considered theoretically and shown experimentally the potassium concentration in the 'fibre water' is given by the equation (7)

$$k_1 = \frac{1}{2}c - (\eta - \epsilon)/2V,$$

and since the second term on the right is negligible since η is almost identical with ϵ ,

$$k_1 = \frac{1}{2}c.$$

If we are dealing with a solution such as Ringer's fluid or the Barkan modification, the total concentration is almost made up of univalent electrolyte, in particular sodium chloride and bicarbonate, with potassium chloride, when this is included. We have then

$$\Sigma d = \frac{1}{2}c,$$

where Σd is the external concentration of the diffusible anions. It appears therefore that

$$k_1 = \Sigma d. \quad (22)$$

From the Donnan equilibrium we have

$$k_1 \times d_1 = k \times d,$$

so that

$$k = \Sigma d_1. \quad (23)$$

We have then these curiously simple relations that the potassium concentration in the fibre water should equal the concentration of total diffusible anion outside and the external potassium concentration should equal the diffusible anion concentration within. (These apply, of course, only for the type of solution considered, or a solution resembling plasma.)

How these agreements hold may be seen from Table 7, in which the data correspond to the experiments of Table 4. Between the range of

TABLE 7
Concentrations of

External potassium k	Internal diffusible anion Σd_1	Internal potassium k_1	External diffusible anion Σd
3	8.3	91	91
6	8.3	92	94
12	11.2	101	100
18	18.1	107	106
30	27.7	120	118
60	65.8	142	148
90	92.0	184	178
120	121.1	212	208
150	150.6	240	238
210	194.4	282	298
300	317.9	353	388

12-210 m.equiv. external potassium, the results confirm very well the validity of the principles discussed.

It is true that the bicarbonate concentration has been calculated from the chloride results on the assumption that it acts similarly, but the mean value of the bicarbonate concentration so calculated is only 5% of the chloride, so that comparatively large differences from chloride—which would certainly be unexpected—would make but little difference to the results.

Why the muscle with 3 or 6 m.equiv. external potassium should show more chloride than the theoretical, will be considered later.

(f) *Potassium accumulation, chloride changes and the Donnan equilibrium*

For the theoretical relations which describe the experimental results in the last section the Donnan relation was used, and since in Table 7, k and Σd_1 as also k_1 and Σd are each equal within such error limits as may naturally be expected, then it follows that $\Sigma k \times d = \Sigma k_1 \times d_1$.

Here, however, we may confine ourselves directly to the chloride analyses. We should expect the relation

$$k \times Cl = k_1 \times Cl_1,$$

(Cl and Cl_1 being the chloride concentrations), and Table 8 (assembled from the experiments of Table 4) shows how it is obeyed.

TABLE 8

External potassium conc.	$k \times Cl$ ($\times 10^{-3}$)	$k_1 \times Cl_1$ ($\times 10^{-3}$)	Ratio of $k \times Cl$ to $k_1 \times Cl_1$
3	0.24	0.68	0.36
6	0.49	0.66	0.74
12	1.05	1.00	1.05
18	1.69	1.72	0.98
30	3.18	2.99	1.06
60	8.16	8.61	0.94
90	14.9	15.8	0.94
120	23.5	24.2	0.97
150	33.9	34.4	0.98
210	60.0	52.8	1.14
300	112.8	118.7	1.05
		Mean	1.01
		Median	0.99

With external potassium concentrations greater than 6 and ranging up to 300 m.equiv./l., the average ratio (median value) of the products of potassium and chloride within and without the fibre is 0.99. The figures for each particular potassium concentration differ no more than may be expected from the sampling error, which will be compounded both of potassium and chloride deviations from their true means.

We have used here the analytical or stoichiometric concentrations, but the equilibration will relate to the products of the activities. From the tables throughout, data are available for a reasonably good estimate of the ionic strengths (apart from protein) within the fibre and hence of the activities. At 12 and 210 m.equiv. of potassium in the external Barkan solution with a constant sodium as described, and a calculated pH of 6.4 and 7.2 within (following the potassium ratio) the square root of the ionic strength ($\sqrt{\mu}$) is 0.40 and 0.61 respectively, and the outside values are 0.32 and 0.55. Applying the equation for the activity (f) of monovalent ions in the form

$$-\log f = \frac{0.5 \sqrt{\mu}}{1 + 1.65 \sqrt{\mu}} \quad (24)$$

(in which in accordance with Cohn's assumption [1927] the mean ionic diameter is taken as 5×10^{-8} cm.), and inserting the above values of $\sqrt{\mu}$ we get the activity of potassium or chloride within to be 0.76 and 0.71 at the low and high k values, and 0.79 and 0.72 outside, from which it follows that the ratio of the stoichiometric products should be 0.93 and 0.98 respectively or a mean throughout of 0.96, which does not differ significantly from the above results.

Calculation of the total muscle chloride concentration from values of the external solution. The mean chloride content of the normal sartorius (reckoned per kg.) is 14 % of the plasma value, and as we have seen almost 13 % is outside the fibres. When the muscle is immersed in Ringer solution, or even in a solution copying the mean electrolyte composition of frog's plasma as closely as possible, with additional glucose to make the osmotic pressures balance, and carbon dioxide to secure the required pH, there is a rapid increase of chloride in the muscle to a higher level of 27 % of the external concentration [Conway & Kane, 1934*a, b*; Fenn, Cobb & Marsh, 1934], the process being complete in about 15 min. If the muscle is immersed in the cold and left over for 24 hr. the increase is much less, rising from 14 to 19.6 %. The significance of such increase will be considered more fully later. Here, it may be noted that, keeping to the experiments in the cold, when the external potassium concentration is raised (with constant sodium concentration) the chloride content of the muscle scarcely alters until the potassium concentration reaches 6-12 m.equiv./l. (Table 7), when it is brought into the theoretical curve of increase with increasing potassium. The general nature of the process will appear from Fig. 5, in which the concentration of total diffusible anion (chloride plus bicarbonate, the latter calculated but in very small proportion) and the potassium concentration of the fibre water is plotted

against the external potassium concentration. It will be seen that the curve of rising anion concentration (almost all chloride) is a straight line passing through the origin, at which point the muscle potassium concentration is still quite high, being close in fact to the normal value. In other words, as the potassium disappears from the external solution so does the chloride from the muscle fibre, in accordance with the slightly extrapolated curve. This extrapolation to the origin, however, will involve

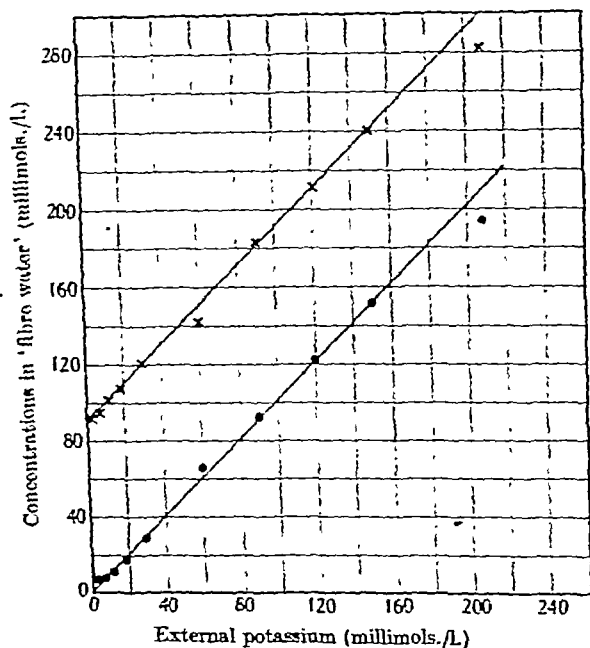


Fig. 5. Mean values of potassium (upper curve) and chloride concentrations in 'fibre water' against the potassium concentration in the external solution containing constant sodium concentration (85.9 mequiv./l.). The lines give the theoretical relation. Immersions for 24 hr. at 2-3° C.; conditions as for Table 4.

among other things a membrane potential reaching indefinite heights (theoretically infinite from the simple equilibrium equations). The deviation of the first few values for the chloride in the excised muscle, and involving only a very small section of the whole curve, may be related to the high membrane potentials necessary to maintain uniformity, and the inability to sustain them in the altered conditions of the muscle environment. As the membrane potential is lowered by the rising potassium the chloride concentrations fall into the general theoretical curve.

We may now consider how the total chloride content per kg. muscle may be calculated theoretically from data on the external fluid. From the Donnan equilibrium

$$k_1 \times Cl_1 = k \times Cl$$

or

$$Cl_1 = \frac{k \times Cl}{k_1} = \frac{2k \times Cl}{c}, \quad (25)$$

from equation (15), the values on the right referring to the external fluid, and Cl_1 the chloride concentration in the fibre water.

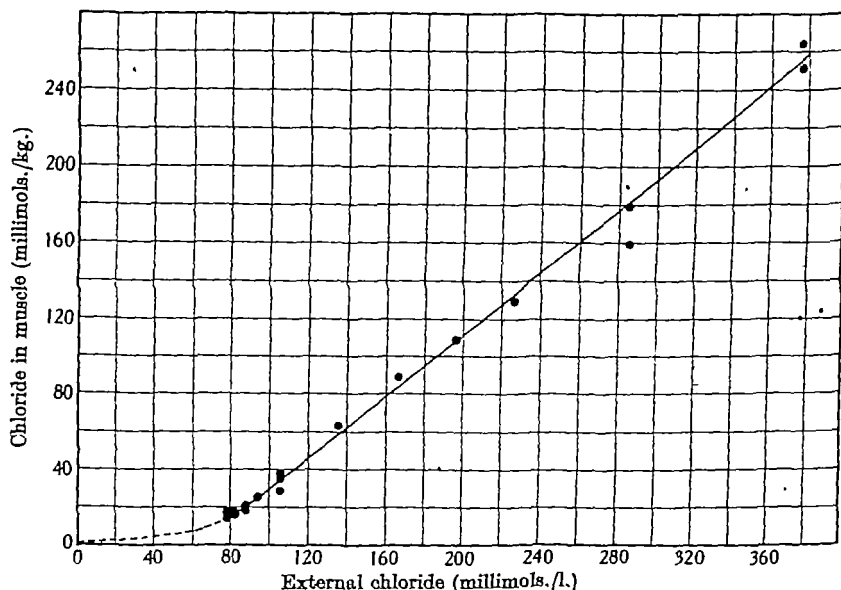


Fig. 6. Total chloride concentration in sartorii (m.equiv./kg. original weight) against the chloride concentration in the external fluid. Immersions for 24 hr. at $2-3^{\circ}\text{C}$., conditions as for Table 4. The line gives the theoretical relation from equation (26) in text, the dots giving the results of single experiments. The increase of chloride concentration in the external solution is due to added KCl, the sodium concentration remaining constant (85.9 m.equiv./l.).

We may write, then, in a similar way to equation (18)

$$C_m' = (0.67 + a) \times Cl_1 + 0.13Cl,$$

which from equation (25)

$$= (0.67 + a) \times \left(\frac{2k \times Cl}{c} \right) + 0.13Cl. \quad (26)$$

Here C_m is the m.equiv. chloride/kg. muscle, and a the weight increase on immersion. This equation gives the total concentration in the immersed muscle (per kg. original weight). Fig. 6 shows the results for a series of

experiments in the cold under the same conditions as described for Table 4, the sodium concentration of the external fluid being constant but with a wide range of potassium concentrations, the muscle volume changing but little. The line is the theoretical line from the above equation.

It is of interest to note that the plotted points in Figs. 3 and 6 for potassium and chloride in muscle are the immediate experimental data without deduction for interspace content or other such allowance, and the lines are the theoretical lines based on the principles discussed.

(g) Potassium accumulation and the membrane potentials

Since the muscle membrane is permeable to potassium and chloride ions it follows that the potential across it will be of the Donnan and not of the diffusion type. It is important that the difference between these be considered, since the assumption that the potential is of the diffusion kind—implying anion impermeability—has been the main cause of the large discrepancies found between theory and experiment.

As is well known, the diffusion potential at the liquid junction of two different concentrations of a salt with univalent ions is given by

$$E = \frac{u-v}{u+v} \frac{RT}{F} \ln \frac{c_1}{c}, \quad (27)$$

where u and v are the mobilities of the anion and cation respectively. With a membrane impermeable to anions v is zero and the potential becomes

$$E = \frac{RT}{F} \ln \frac{c_1}{c}. \quad (28)$$

When there is a Donnan equilibrium across the membrane permeable to both ions the potential is given by the same equation, but an important difference lies in the effect of increasing the external concentration, as, for example, increasing the potassium concentration outside muscle, the volume of which is maintained constant. With anion impermeability this should have no effect on the internal potassium concentration, but in accordance with the equilibria considered above, it will have a very marked effect.

The potential across the muscle membrane, from the potassium equilibrium is given by

$$\pi = \frac{RT}{F} \ln \frac{k_1}{k} \quad (28a)$$

$$\begin{aligned} &= \frac{RT}{F} \ln \frac{c}{2k} \text{ (from equation (15))} \\ &= 58 \log c/2k \text{ (millivolts at } 18^\circ \text{ C.),} \end{aligned} \quad (28b)$$

π being positive in the direction from within out. From this equation (given also as equation (13*b*)) we should obtain the maximum potential difference available for injury currents with one contact on the muscle surface and the other at the cut end. For each observation the same solution is used for the two contacts, but the potassium concentration varied as desired from one experiment to another.

When both contacts are on the intact muscle surface (each contact being made in this case with a different solution) the potential difference at room temperature (18–20° C.) is given by

$$\pi = 58 (\log c_0/2k_0 - \log c/2k), \quad (29)$$

k_0 and c_0 being the external potassium concentration and the total concentration at any one contact, and k and c the concentrations at any other

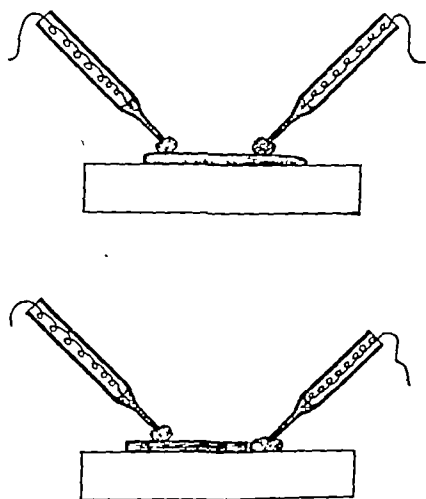


Fig. 7. Contacts with muscle on paraffin block, showing position of filter paper wads soaked in the contact solution and the chloridized silver wire.

contact. (In equation (29) no account is taken of potential differences which may possibly arise between electrodes immersed in different solutions apart from the potential differences at the membrane surfaces. Such may be allowed for as described below.)

Method of investigation. The potentials were investigated on the sartorii of curarized frogs, usually within 1 hr. after the curare injection. (Rather marked diminution was noticed if the observations were delayed for 3 hr.) The muscle was excised, its surface dried with filter paper and placed on a small dry paraffin block (about 30 × 10 × 5 mm.). With a fine bent forceps a small wad of filter-paper pulp was removed from a large volume of the contact solution in which it was being soaked, the excess fluid was squeezed out and the wad placed across the muscle near one end; a wad similarly prepared was placed towards

the other end. If the potential differences between intact and injured surfaces were being investigated, the muscle was cleanly sectioned, the second was placed on the block and the muscle moved so that contact was made with its cut end (vide Fig. 7). For this latter investigation the solutions at both contacts were the same. With the muscle in position the paraffin block was lowered into a small compartment cut out in a large wooden block and lined with paraffined walls, such as could be sealed off from the external atmosphere if necessary by a glass slide. The electrical contacts were of a kind shown in Fig. 7, non-polarizable electrodes of chloridized silver dipping into contact fluid held by capillary attraction in a drawn out glass tube. The remaining apparatus has been described under 'Methods'. Where different contact fluids were used, as in measuring the potential difference between two intact surfaces, allowance was made for the potential difference arising at the electrode contacts with the solutions (apart from the muscle membrane potential difference) by a separate determination after each muscle experiment. In this determination the electrodes were immersed in their respective contact solutions contained in small beakers, the solutions being connected by a capillary agar bridge containing saturated KCl.

The contact fluids were similar to the Ringer fluid of Barkan type already described, being designed as in § IV (c) to maintain a constant muscle volume with wide changes of potassium concentration. Apart from the added KCl the solutions had the same total concentration as in that section, but bicarbonate was omitted (and also carbon dioxide), the phosphate concentration being increased to 3 mM/L, and sodium chloride added to make the balance. Thus with varying potassium concentration there was a constant sodium concentration and, as already shown, this procedure suffices to keep the muscle volume largely constant with great increases of potassium concentration.

Potential differences between two surface contacts. One contact solution was maintained constant throughout with a potassium concentration of 30 m.equiv./l. while the other was varied. Table 9 gives the theoretical and the experimental results, compared also with the potentials to be expected from anion impermeability. Between 12 and 300 m.equiv./l. potassium concentration outside, the total difference on this latter view should be 81 mV., but it should be only 48 mV. according to the theoretical basis of the present discussion, or from equation (29), and if we were to substitute in equation (28a) the potassium concentrations in the muscle (k_1) found experimentally after 24 hr. equilibration, we should obtain 50 mV. difference. The actual mean difference observed was 47 mV., as given in Table 9.

The maximum potential we could obtain between the variable contact and that of fixed composition (allowing for the potential difference between electrodes and solutions) was 20 mV. (mean value), which with the theoretical value of 35 mV. across the membrane at the fixed contact should result in 55 mV. for the maximum value of the injury potential, the actual observed mean value being 59 mV. We were at first much surprised at the close correspondence between the theoretical and the actual potentials throughout, since it would be expected that appreciable short-circuiting would occur. We sought therefore to remove possible

TABLE 9. Potential differences between two surface contacts, one with fixed potassium concentration

Potassium conc. in variable contact solution mM./l.	Total conc. of variable solution mM./l.	Theoretical values for		Experimentally observed muscle potentials (allowing for P.D. between the electrodes at electrode-solution contacts)
		Donnan equilibrium potentials. Full theoretical value (eq. (29))	Diffusion potentials from anion impermeable view	
0	185	—	—	-20 (3)
12	209	-19	-23	-17 (3)
18	221	-11	-13	-11 (2)
30	245	0	0	0
60	305	+10	+17	+11 (3)
90	365	+17	+28	+18 (2)
150	485	+23	+41	+23 (2)
300	785	+29	+58	+30 (3)
Total P.D. between 12 and 300 m.equiv./l. external potassium concentration.				
		48	81	47
Injury potentials				
0	185	—	—	-59 (4)
12	209	-55	-59	-57
18	221	-46	-49	-46
30	245	-35	-36	-39
60	305	-24	-19	-30
90	365	-18	-9	-23
150	485	-12	+4	-14
300	785	-7	+22	-6 (2)
Total injury potential between 12 and 300 m.equiv./l. external potassium concentration.				
		48	81	51

(The figures in brackets in the last column refer to number of determinations.)

shorting by the electrolytes of the interspace fluid, by stirring for 30 min. in isotonic glucose with just a trace of potassium (2.5 m.equiv./l.). Under these conditions we know for certain that 95-100 % of the true interspace electrolyte will have diffused outwards from frogs' sartorii. If appreciable shorting were present due to the interspace solution it should be evident on subsequent examination of the contact potentials. We found no evidence of such shorting, and were forced to conclude that under the conditions of our working it was negligible.

Potential differences between injured and intact surfaces. When we consider that within the fibre there is a mean potassium concentration of 126 m.equiv./l. of 'fibre water' and 2.5 m.equiv./l. of plasma, we may expect a potential of 99 mV. across the membrane (from equations (11), (13b) or (28a)). This assumes that the potassium is all or almost all ionized, but from the results throughout [vide also Hill, 1931] no other view appears tenable. From the previous section instead of 99 mV. we should obtain only 55 mV. if the basis of our reasoning has been correct.

Experimentally we have found a mean of 59 mV. (4 obs.) under the conditions described, a figure quite close to the expected value and differing perhaps no more than the sampling error. The question arises as to the cause of the difference between this figure and 99 mV., and it may be said that it is not shorting but rather the inability of the excised muscle to retain its normal permeability above a certain potential across the fibre membrane. Under the conditions and with the solutions described, it is not possible to obtain potential differences across the membrane exceeding about 60 mV. This is indeed easy to demonstrate, as we raise the potassium concentration in the contact solution from zero onwards. We get practically no change until the potassium concentration becomes about 10 m.equiv./l. and then the potential begins to fall in accordance with the theoretical equations (13*b*) or (28*a*), as shown in Table 9.

(It may be noted that in the experiments described for potentials between two intact surfaces as also for the injury potentials, equilibrium was reached rather quickly when relatively low potassium concentrations were used, and within 15 min. for all the ranges described.)

Muscle potentials and anion impermeability. It is obvious from the foregoing experiments that so far from the electrical phenomena indicating anion or chloride impermeability (which in any case is clearly disproved by the chemical analyses) they are for the first time quantitatively explained by equilibria based on the opposite view. The experiments of Höber [1904] are often cited in this connexion. With double-contact experiments in which Ringer solution forms the upper contact, and varying isotonic solutions, the lower contact, the lower end of the muscle being immersed, it was observed that little or no variation in potential occurred on varying the anion in the lower solution, but marked differences on substituting certain other cations for sodium. Even if the anions in the lower solution were indiffusible through the membrane, no appreciable change would be observed, because at the Ringer solution junction (upper contact) the maximum potential would exist across the membrane. With sodium and an indiffusible anion in the lower solution, the potential would be again the maximum derived from the potassium and chloride in the muscle diffusing out towards an equilibrium. With sodium and a diffusible anion we should likewise get no difference, the diffusible anion merely substituting for chloride. In short, provided the cations of the lower contact are indiffusible, and disallowing for some specific action, we should get no potential difference between it and the Ringer solution contact, whether the substituted anions be diffusible or not,

because the maximum potential will be sustained across the membrane at both regions.

Examining the mean of the potentials recorded by Höber, of the nine anions used in the lower solution with sodium cations (or lithium and magnesium), sulphocyanide was exceptional throughout, showing 9.5 mV. positivity against the Ringer solution. Sodium nitrate also gave 5.0 mV., but the remaining seven sodium salts did not give higher than 2.5 mV. difference in the mean, and five gave zero. On the whole, this shows a good agreement with the expected result, considering that the potential at either contact is about 60 mV. It may be added that when isotonic potassium chloride (and to a lesser extent ammonium and rubidium chlorides) was substituted for sodium salts in the lower contact solution a marked negativity was observed, which accords with our views and experiments as described above.

POTASSIUM LOSSES, AND ASSOCIATED CHANGES, FROM MUSCLES IMMERSSED AT ROOM TEMPERATURE IN RINGER FLUID WITHOUT RAISED POTASSIUM CONCENTRATION, AND THE MAINTENANCE CONCENTRATION OF POTASSIUM

Apart from the immediate bearing on the potassium content of muscle these changes are important for any consideration of the pH and original bicarbonate concentration when an attempt is made to determine these through bicarbonate/carbon dioxide equilibria [e.g. Stella, 1929; Fenn, 1928].

Immersion in three different solutions was investigated; the ordinary Ringer-Locke fluid containing 1.9 m.equiv. potassium/l., the Barkan modification as described under 'Methods', containing 1.3 m.equiv. potassium/l., and the special fluid also described there resembling the electrolyte content of frog's plasma as closely as possible from the more recent analyses, but with glucose addition to make the osmotic balance. Muscles immersed in these solutions were examined for chloride, sodium and potassium changes as judged from a comparison with fresh companion tissues (usually four muscles from four frogs being used for one immersion experiment). Chloride changes in immersed *sartorii* have been already investigated [Conway & Kane, 1934*a, b*; Fenn & Cobb, 1934; Conway & Cruess-Callaghan, 1937]. It was shown that the chloride content of 100 g. muscle increased from a mean value of 14.5 % of the external concentration to 27 % (or to 33.5 % reckoning on the total water content of muscle), the chloride entrance being nearly complete in 15 min., and showing after this only a very slow continuous rise at room temperature.

As will appear from Figs. 8-10 (curve *B*) sodium enters more slowly at first than chloride (curve *A*), and this difference is most marked for the immersions in the special fluid (Fig. 10), when after 5 min. there is

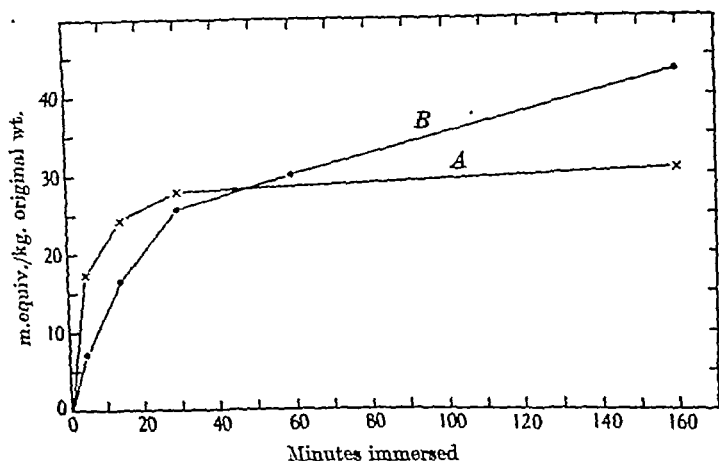


Fig. 8. Mean gains of chloride (*A*) and of sodium (*B*) for sartorii immersed for 2 hr. at room temperature with oxygen bubbling in Ringer-Locke solution which contained 0.71 % NaCl, 0.1 % glucose and the usual potassium, calcium and bicarbonate concentrations. The points give each the means of two to six experiments using four muscles at a time.

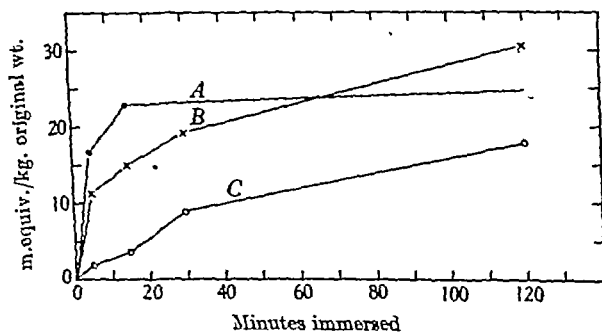


Fig. 9. Mean gains of chloride (*A*) and of sodium (*B*) and loss of potassium (*C*) for sartorii immersed for 2 hr. at room temperature with oxygen (97 %) and carbon dioxide (3 %) bubbling in solution of Ringer-Barkan type containing 0.7 % NaCl and 1.3 m.equiv. potassium/l., the remaining composition being described under 'Methods'. Two to six experiments for each point.

a scarcely perceptible change in sodium content, but most of the extra chloride has already entered. (The points in Figs. 8-10 are the means of two to six experiments for each time period.) After a time (45-120 min.) the sodium increase exceeds the chloride, and the entering sodium con-

tinues to rise roughly parallel with the potassium loss (curve C). From the beginning of the immersion there is a continuous emergence of potassium from the sartorius as shown in Figs. 9 and 10. Since the sodium and chloride concentrations in the Ringer-Locke and Ringer-Barkan fluids are greater than those in plasma or in the special fluid (the sodium concentrations for the three being 124, 133 and 104 m.equiv./l.

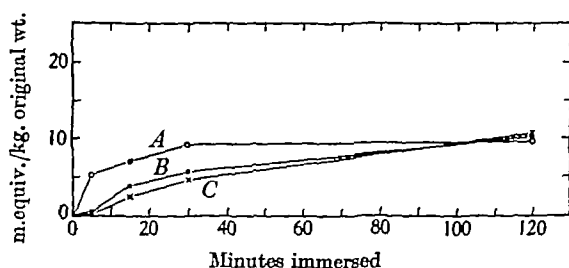


Fig. 10. Mean gains of chloride (A) and of sodium (B) with loss of potassium (C) for sartorii immersed for 2 hr. at room temperature in the special fluid closely resembling the electrolyte composition of frog's plasma (described under 'Methods'). Bubbling with oxygen (97 %) and carbon dioxide (3 %). Two to six experiments for each point.

and the chloride concentrations 130, 124 and 74), some of the increased sodium and chloride content will be accommodated in the interspaces. Allowing 13 % of the excess over the plasma value for this, and with the knowledge that in 30 min. equilibrium will have been attained between such spaces and the external solution, we have made out Table 10, showing

TABLE 10. Gain of Na and Cl by fibres and loss of K, allowing 13 % of muscle weight as effective interspaces

Solution	Changes after 30 min.			Changes after 120 min.		
	Na	K	Cl	Na	K	Cl
Ringer-Locke	23.0 (5)	—	18.4 (4)	35.9 (6)	—	21.0 (2)
Ringer-Barkan	15.3 (2)	- 8.9 (2)	18.1 (2)	26.4	- 17.7	19.3 (2)
Special	5.7 (2)	- 4.8 (3)	10.4	10.5	- 10.9	9.8 (2)

The Ringer-Locke solution was made up on the usual Ringer basis with 0.1 % glucose, and containing 0.71 % NaCl. The Ringer-Barkan fluid contained 0.70 % NaCl, the remaining composition being as described under 'Methods', where the composition of the special fluid is also given.

the amounts of sodium and chloride which have entered the fibres in 30 and 120 min. respectively. Thus, with the Ringer-Barkan fluid, the fibres gain (per kg. muscle) 15 m.equiv. sodium and lose 9 m.equiv. potassium in 30 min., and they gain 26 m.equiv. sodium and lose 18 m.equiv. potassium in 120 min. With the special fluid these changes are much less, the sodium gain being about equal to the potassium loss,

and with the Ringer-Locke they are more. The chloride content shows very little change from 30 to 120 min., and the total increase goes approximately proportional to the external chloride concentration, being about 14 % of this (reckoning per kg. muscle), which with the 13 % in the spaces gives a total of 27 % (or 34 % when calculated on the total water in muscle, a calculation previously defined as the 'permeation' of chloride and corresponding with the results then obtained). The significance of such changes at room temperature are again referred to in the Discussion.

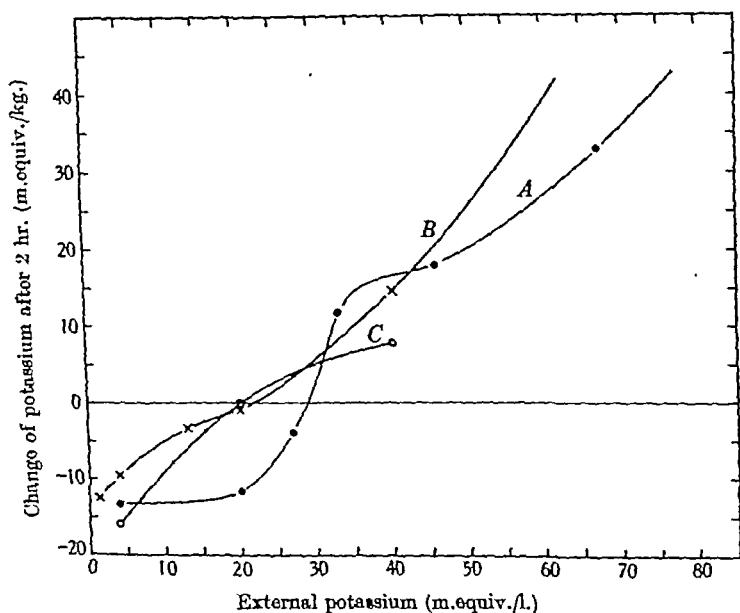


Fig. 11. Mean changes in potassium content of the frog's sartorius (*A*), kidney (*B*) and liver sections (*C*) immersed in Ringer-Barkan solution for 2 hr. at room temperature with oxygen (97 %) and carbon dioxide (3 %) bubbling. The solution had a constant total composition (243 mM./l.) but varying potassium content. Liver sections cut to about 1 mm. thick. Two to six experiments for each point. Tissues weighed before immersion.

The maintenance concentration of the external potassium. The plasma concentration of potassium naturally prevents potassium loss in vivo, but after excision of the sartorius and immersion in Ringer fluid, the maintenance concentration, or the concentration required for neither loss nor gain of potassium, changes considerably, and this not only applies to muscle but also to other organs such as the kidney and liver, the latter investigated as sections. At room temperature and using Ringer-Barkan

We may take it that the potassium considered enters almost altogether as the chloride and consequently in the ionized condition. This amounts to saying that the muscle membrane is both cation and anion permeable, but at the same time permeable only to a certain limit as shown by the difference between potassium and sodium and by the organic phosphate anions and chloride.

The cation limit cannot be better defined at present than by placing it between potassium and sodium, but that some numerical precision can be given to this will appear below. The anion limit under the conditions of raised external potassium concentration may be deduced from an examination of the results of Overton [1904]. He showed that the sartorius muscle swelled when immersed in isotonic bromide, iodide, chloride, nitrate, but that it did not swell in potassium phosphate, sulphate, acetate, tartrate or ethylsulphate. The division into these two series suggested to Höber [1922] a connexion with the Hofmeister series, and that the swelling was due to some effect on the proteins of the interstitial tissue (?) an explanation which is definitely disproved by the results here described. We may now consider what appears to be the true explanation of this division. The mobilities of the ions under uniform gradient of 1 V./cm. (or 0.5 V./cm. for divalent ions) may be used to give a comparative measure of the ion size. Assuming that the ions with their associated water molecules can be treated as spheres, then from the equation of Stokes the velocities will be inversely proportional to the radii or diameters. Table 11 gives a list of the mobilities and the relative

TABLE 11

Velocities of ions under gradient of 1 V./cm. or 0.5 V./cm. for divalent ions				Relative ion diameters (diameter of potassium ion = 1.00)			
Cations		Anions		Cations		Anions	
H	315.2	OH	173.8	H	0.20	OH	0.37
Rb	67.5	Br	67.3	Rb	0.96	Br	0.96
Cs	64.2	I	66.2	Cs	1.00	I	0.97
NH ₄	64.3	Cl	65.2	NH ₄	1.00	Cl	0.98
K	64.2	NO ₃	61.6	K	1.00	NO ₃	1.04
Na	43.2	CH ₃ COO	35.0	Na	1.49	CH ₃ COO	1.84
Li	33.0	SO ₄	34.0	Li	1.95	SO ₄	1.89
Ca	25.5	HPO ₄	28	Ca	2.51	HPO ₄	2.29
Mg	22.5			Mg	2.84		

ion diameters, the mobilities being taken from the *International Critical Tables* and the *Chemiker Kalender* (the values for the divalent ions being reduced for a half-volt gradient, since the listed figures for the divalent ions, though expressed, for example, as $\frac{1}{2}\text{SO}_4$, etc., give the true velocities of the ions for 1 V./cm.).

It will be seen that the line dividing the diffusible ions from the indiffusible (assuming H and OH as diffusible) also divides the two series into groups below and above a certain ion diameter. The explanation of the permeability or alternatively that of the muscle swelling in certain isotonic solutions appears obvious therefore from the theory of a molecular sieve. If the solute of the isotonic solution in which the muscle is immersed yields a cation with diameter of about 1.2 or less (referred to potassium as unity) or an anion with diameter of about 1.4 or less, the muscle will swell because salt will enter and the volume will increase in accordance with the theoretical treatment as previously outlined. If one or both of the ions has a diameter greater than this the muscle does not swell. (Phosphate permeability in vivo appears exceptional.)

The similarity of level here of anion and cation diameters for diffusion through the membrane, suggests the view that the same molecular pore exists for both and that this is probably not charged within the membrane. From other experiments, however, it would appear that marked differences may occur between the anion and cation permeability with respect to ion diameter.

Permeability with raised potassium concentration

We have already considered that a change of permeability occurs as we increase the potassium concentration in Ringer solution, but that the change is an improvement since sodium is better kept out (in fact perfectly over 24 hr. in the cold) and so approaches closer to what must be the normal condition. Not only is there a narrowing of the cation pores as we pass on to potassium concentrations that are in true equilibrium with the internal potassium, but there is also a narrowing of the anion pores and this would seem to exclude even chloride at extremely high salinity, of the order of 10% KCl. Over a wide zone of increasing potassium concentration we have a condition which must simulate the normal intact muscle rather closely, with perhaps a more restricted anion permeability; thus chloride, bromide, nitrate, etc., will be allowed to pass but not phosphate.

Permeability in Ringer solution or similar fluid with normal plasma potassium

The question arises as to why there is defective permeability in the sartorius muscle in Ringer solution and an improvement towards normality as we increase the potassium concentration. This has been largely answered under the section on resting potentials. It was there noted

that a maximum potential of about 60 mV. only could exist across the membrane of excised muscle (such a figure we have found under the conditions examined, though doubtless it is subject to some change with the nature of the external fluid apart from the potassium content). If the external potassium concentration is below the level corresponding to such a potential, potassium must come out and chloride enter. We may suppose that in such a condition the membrane alters and shows considerable permeability differences from the normal. This explains what happens when the excised sartorius is immersed in Ringer, Ringer-Barkan or other such solution. As shown in the previous section there is then an increase in anion permeability characterized by a sudden inrush of chloride coming practically to an end within 15 min. This is accompanied by an increase in cation permeability with entrance of sodium, potassium being lost steadily, the quantities involved being described in that section. The significance of these changes will be better appreciated when we consider the maintenance concentration of potassium and its relation to the chloride changes.

The significance of the maintenance potassium concentration and its quantitative relation to the chloride change in the immersed sartorius

If the muscle membrane we have been considering were of a perfectly stable kind there is no reason why potassium should *continue* to be lost even if the external potassium concentration were reduced to zero. A comparatively very small quantity corresponding to the chloride and bicarbonate in the fibre water (or about 2.5 m.equiv./l.) would emerge, but the remainder would be held by the electrostatic attraction of the indiffusible anions. We could explain the loss in one way by supposing that the indiffusible anions were breaking down to anions of diffusible dimensions. Thus, phosphocreatine may be breaking down to phosphate which we could consider diffusible, and to creatine. The outward diffusion of potassium is however much more rapid than this breakdown would warrant, and, moreover, we can prevent the steady loss of potassium by raising the external concentration to only a fraction of the total potassium concentration inside the fibre. The potassium concentration in the cold can be stabilized in this way even for 48 hr., and, as previously shown, there is no appreciable loss of anions. The true explanation of these occurrences appears to be that already proposed, that the muscle can stand only a certain concentration difference across it. When this is exceeded the membrane system breaks down and hitherto indiffusible cations enter and anions are lost, until a new level is established. The

highest concentration differences and potentials can be withstood *in vivo*, but much less in the excised and immersed muscle. This idea not only explains the loss of potassium, the entrance of sodium, and the constancy of the potential differences between injured and intact surfaces until the potassium in the contact fluid exceeds a certain level, but as we shall see, it accounts *quantitatively* for the rapid chloride entrance in the sartorius muscle when immersed in Ringer solution.

The diffusion of chloride through the membrane is much more rapid than that of potassium, and chloride enters quickly in exchange for the internal anions, the chloride ratio falling until it reaches the level the membrane can withstand. Subsequently it enters at a comparatively negligible rate. As we increase the external potassium concentration the chloride will remain at this level or ratio until the maintenance concentration of the potassium is reached (vide Table 4), the chloride ratio then falling with further rise of potassium concentration, in accordance with the theoretical equations.

Turning to the data of Table 10 it appears that after 2 hr. at room temperature in Ringer-Barkan solution, 19.3 m.equiv. chloride have entered the fibres in 1 kg. muscle, or 19.3/0.67 in a litre of fibre water. The concentration ratio is therefore 124/28.8 or 4.3 (the external chloride being 124 m.equiv./l.). Corresponding to this we find that the potassium ratio at the maintenance level is 126/30 or 4.2 (in the reverse direction to the chloride), which is in good agreement.

Similarly, if we consider the 24 hr. immersions in the cold from Table 4 the chloride in the fibre water with an external potassium concentration of 3 and chloride concentration of 79 m.equiv./l. is 7.2 with a chloride ratio of 79/7.2 (=11.0), and again at an external potassium concentration of 6 and chloride concentration of 82 it is 7.2 with a ratio of 11.4. We have seen that the maintenance concentration in the cold is 11 corresponding to a potassium ratio of 126/11 (=11.4), which gives also a good agreement with the chloride ratio. It is obvious that the maintenance concentration may be regarded as in equilibrium with that in muscle and the equivalence of the products of potassium and chloride concentrations within and without should then hold in accordance with the Donnan relation. This is clearly so from the above data, just as it continues to hold with a potassium concentration increased to over 20 times the maintenance level (vide Table 8).

Fig. 13 illustrates the maintenance concentration of potassium for excised muscle and its relation to concentration ratios across the membrane. The curve *HCFG* gives the concentration ratio of potassium *in vivo*

with change in the external potassium concentration, the sodium chloride being maintained constant at the level required to ensure no change in volume (with the Barkan solution described under 'Methods', and using excised muscle this NaCl concentration will amount to approximately 88 mM./l.). The region *CFG* of the curve may be taken as corresponding to the experimental findings for excised muscle.

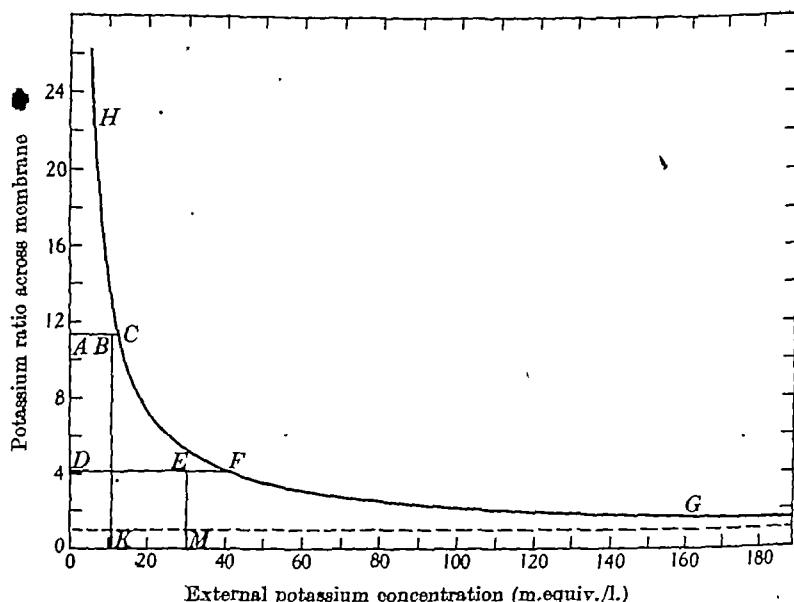


Fig. 13. Diagram showing curve (*HCFG*) of the equilibrium concentration ratio of potassium across the muscle membrane with varying potassium concentration outside, but constant sodium concentration maintaining constant muscle volume. Points *K* and *M* represent maintenance concentrations of potassium for immersions in the cold and at room temperature respectively.

For muscle in the cold the maintenance concentration of potassium, namely 11 m.equiv./l. corresponds to a concentration ratio of 11.4 or the line *AB*. For concentrations below *B* or *K* the muscle will lose potassium to maintain the ratio as at *B*, which is a maximum under the conditions. Beyond *B* the muscle will gain potassium, but for a short distance the ratio will not change until the external concentration reaches *C*, beyond which the muscle will continue to gain potassium but with a falling ratio. Similar considerations apply to the maintenance concentration at *M* or *E* for room temperature (2 hr. experiments).

It will be seen that the potassium entering the muscle from *C* or *F* onwards does so against a gradient.

When the sartorius is immersed in Ringer solution with the usual potassium concentration the widening of the anion pores permits many anions to enter besides chloride, which may be normally indiffusible across the membrane, e.g. sulphate, gluconate, and even ferrocyanide. The diffusion coefficients for the entrance of these anions we have found to be practically identical within the sampling error and this may be explained theoretically. (The similarity of the diffusion coefficients of the anions in muscle has been criticized by Eggleton *et al.* [1937] but meanwhile we have carried out a large series of determinations on six different kinds under strict controls and our earlier conclusions have been fully confirmed.)

It may also be noted that during the period of increased permeability not only anions of greater size may enter the fibres, but it is also possible that neutral molecules normally impermeable may pass the membrane. It has been shown, however, that at least the divalent magnesium cation does not penetrate appreciably (apart from its entrance into the inter-fibre spaces) within the short period in question, and, as may be expected, inulin would appear strictly debarred over long periods.

Eggleton *et al.* [1937] have attempted to explain the increase of chloride on immersion as due to an adsorption on the fibres. This is invalidated if only by the fact that no appreciable increase of sodium need accompany the chloride increase, as noted above (vide Fig. 10), so that the chloride must interchange with anions inside the fibre. Their explanation was advanced also in the belief that the membrane is essentially anion impermeable; but the evidence presented here makes such a view untenable.

In Ringer or Ringer-Barkan solution we have then at room temperature a defective membrane permeability with the plasma level of potassium. It requires a relatively high external potassium content to prevent potassium leaving muscle—as high as 29 m.equiv./l. In the cold the defect in the membrane is not so marked, the external maintenance concentration being smaller (about 10–12 m.equiv./l.). We have considered these results to be associated with maximum sustainable potentials, and may now consider an interesting comparison between such immersed muscle data and the experiments of Mond & Netter [1932] in which the hind-limbs of frogs were perfused with Ringer fluid introduced through the aorta. Very little disturbance of the normal membrane conditions appears to result from this procedure. Potassium enters the muscle if it exceeds only 3.4 m.equiv./l. in the perfusing fluid, sodium does not appreciably increase and, as might be expected, *there is little or*

no rise in chloride. In such perfusions perhaps the most significant difference from the immersed muscles lies in the proportion of the total perfused fluid to the amount of tissue, for in the perfusion experiments the volume of the perfusion fluid was about the same order as the total muscle weight, and some substance in the plasma or interspace fluid necessary perhaps for the normal maintenance of the membrane functioning would be less diluted than for the immersed muscles.

The results of Fenn & Cobb [1934] with comparatively high phosphate concentration, have been considered in a previous section.

The permeability of the normal muscle in vivo

The permeability of the cell membrane in the living organism has been recently investigated by the use of radio-potassium, radio-sodium, etc. For voluntary muscle in the rat Greenberg *et al.* [1938] found 4 hr. after injection the ratio of the radio-potassium in plasma to that in a similar weight of tissue to be 1.0/0.6, the amount in muscle being therefore far more than could be attributed to the interspaces. Hahn *et al.* [1939] found a higher ratio of 1.0/1.3 for the frog, 1 hr. after injecting radio-potassium, and 1.0/1.45 after 24 hr., obtaining similar figures for the rabbit. With radio-phosphate the ratio was 1.0/0.60 and with radio-sodium 1.0/0.085 in the rabbit's gastrocnemius, which latter corresponds to what may be expected with radio-sodium present only in the inter-fibre spaces. It is clear therefore that the muscle membrane must be permeable to phosphate and potassium but not to sodium.

The high phosphate value for the muscle is no doubt due to the inorganic anion being incorporated as phosphate esters. What is peculiar, however, about this penetration of radio-potassium into muscle is that it amounts to only 5 % of the expected amount if it interchanged freely with the whole of the muscle potassium. Such a result could be explained if we supposed only a small fraction of the muscle potassium to exist as the free cation, but the evidence is so strong against this that another explanation must be sought. We could suppose that the radio-potassium diffuses into some outer compartment of the muscle which contains relatively little of the muscle potassium, and for which view the anatomical structure of the fibre might be considered to lend some support. Since, however, the radio salt appears to act in a similar way with other tissues, this again does not seem a likely interpretation. Further, it may be suggested that the permeability of the cell membrane is poised at a certain potential level across it, so that if this falls the permeability diminishes and if it rises the permeability increases. Increase of potas-

sium in the blood will lower this potential and may do so quite markedly. Thus an increase of 50 % in the plasma potassium representing a small absolute figure may be expected to change the potential across the membrane in rabbit muscle by as much as 11 mV. A narrowing of the pores with falling potential may account for the fact that the initial rapid inrush of potassium is followed by an extremely slow subsequent entry. A somewhat similar phenomenon may be observed in excised muscle, for as the potential across the membrane falls with rising potassium concentration outside there is a diminished cation permeability.

It is also possible that the radioactive elements themselves have some membrane effect. This question has been recently raised in the *Physical Review* by Barnett [1939] who questioned the indifference of the membrane to short range γ -ray bombardment. Crane [1939] and Mullins [1939] in replying agreed in regarding an upper limit of concentration beyond which such effects may be expected.

Mullins [1939] considers the experiments with radio-sodium such as those of Cohn & Cohn [1939] to be conducted with dosages below this limit, but points out that it varies considerably for different radioactive isotopes and should be determined for each. The question does not appear altogether settled, at least not for radio salts other than sodium.

The membrane equilibria for hydrogen and bicarbonate ions

Since the muscle membrane has been shown to be permeable to potassium and chloride ions it may be assumed permeable to hydrogen, hydroxyl, and bicarbonate ions, so that we should have

$$\frac{k_2}{k} = \frac{[\text{H}^+]_i}{[\text{H}^+]_o} = \frac{[\text{OH}^-]_i}{[\text{OH}^-]_o} = \frac{[\text{HCO}_3^-]_i}{[\text{HCO}_3^-]_o}.$$

Since the values for the bicarbonate ratio (and by inference from this for the hydrogen ion ratio) hitherto obtained, seem opposed to such a relation it is necessary to inquire at some length into the exact meaning of the data presented [e.g. by Stella, 1929, or by Fenn, 1928].

The pH of the excised sartorius. Over the range of potassium concentration investigated in the cold (at constant volume) the pH should change from 6.2 to 7.2 with an external value of 7.3, corresponding to ratios across the membrane of 11.4 and 1.3. Similarly for the muscle immersed at room temperature in the Barkan fluid described, at and below the maintenance level, the pH should be approximately 6.7 with external value of 7.3, and ratio of 4.2 across the membrane.

As to the pH within the fibres in vivo, and considering the potassium concentration ratio across the membrane to be about 50, we should expect

an internal pH of 5.9 with plasma pH of 7.6. This latter figure, as a mean value for frog's blood, was obtained by Barkan *et al.* [1921] using an electrometric method, and would also correspond to Fenn's figures of 25 m.equiv./l. for the plasma bicarbonate, and carbon dioxide tension of 20 mm. If the pH in vivo is 5.9 the change to 6.2 or 6.7 for the immersed muscle will necessitate the entrance or formation of alkali amounting to 5–14 m.equiv./kg. (for calculation see later) and the consideration of this entrance is essential for interpreting CO_2 and bicarbonate equilibria. It could be derived in the following ways.

(1) Entrance of HCO_3^- , HPO_4^{--} or OH^- ions. When the muscle is immersed in Ringer solution with the usual potassium concentration it is unable to maintain the high in vivo concentration or potential. The membrane alters in its pore structure so that diffusible anions enter to replace some previously indiffusible. This anion change goes rapidly and with it sodium enters and potassium is lost. The diffusible anions reach quickly what we may regard as an equilibrium concentration, and corresponding to the maximum ratio the membrane can maintain. As HCO_3^- , HPO_4^{--} or OH^- enter they combine with hydrogen ion within, and the ratio for these ions rises across the membrane, so that a further release of indiffusible anions or entrance of sodium is necessitated, this process continuing until the hydrogen ion concentration ratio corresponds to that of the diffusible anions, or to the figure towards which the potassium is tending.

With regard to the relative entrance of these different anions it is likely that hydroxyl ion will account for only a small fraction of the change, which will be mostly due to bicarbonate or alkaline phosphate ion, depending on the relative concentrations of these in the external fluid. When the potassium concentration externally is raised to the maintenance level or above it, it may be taken that the increasing pH with increasing potassium concentration will be reached by the entrance of potassium bicarbonate (or phosphate, if this is also present externally) without the emergence of previously indiffusible anion. Even with ordinary Ringer solution and the external bicarbonate or phosphate concentration of the order of only a few m.equiv./l., the required entrance of alkali may be expected to occur quickly owing to the rapid anion diffusion compared with the cation. The process probably reaches completion within the hour, but is associated with sodium entrance and loss of anions—indiffusible in vivo.

(2) Direct emergence of the hydrogen ion accompanied with anion or exchanging for sodium.

Owing to the low hydrogen ion concentration this probably occurs only to a small extent, but the possibility may be entertained that a high comparative diffusion rate for hydrogen ion may compensate largely for its low concentration.

(3) Emergence of the undissociated molecules of a weak acid or of the cation of a weak base dissociating within the pH range of muscle.

In this exchange certain amino acids may possibly take part.

It would appear that the entrance of sodium hydroxide may be quite outruled, since, however slowly it penetrated, the sodium would thereby tend to the same concentration ratio as potassium, and no such tendency is noticeable with raised potassium concentration external to excised muscle, no more than it can possibly occur in vivo.

The exact part played by these various exchanges cannot yet be assessed. As mentioned, the total resulting alkali required for the pH change from 5.9 to 6.2 in the cold, or 6.7 at room temperature, is from

5 to 14 m.equiv./kg. muscle. Actually less than this is required to come from the outside fluid, since about 3 m.equiv./kg. of bicarbonate exist in the interspaces of excised muscle. This is immediately available and probably rapidly used after the excision, so that only 2-11 m.equiv./kg. need be derived from further interchanges with the external fluid. From examination of Table 10 it will be seen that for the sartorius in ordinary Ringer-Locke solution, after 30 and 120 min. immersion 23 and 36 m.equiv. of sodium have entered the fibres (apart from the interspaces) with 18.4 and 21.0 m.equiv. chloride, so that changes of the order of 11 m.equiv. alkali in the various ways in which this may enter are certainly not unlikely. Similar considerations apply to the changes with the Ringer-Barkan solution.

Comparison of the pH deduced from the membrane equilibria and other methods. Concerning the pH in vivo the experiments of Rous [1925] are here the most important, for they were carried out with the minimum disturbance of the living tissue. He has shown, using intravital staining in mice, and in particular with the phthaleins, that the reaction of voluntary muscle as well as certain other tissues examined 'would seem to be at least as acid as pH 5.6'. Such experiments 'were made under circumstances which excluded common sources of error, for example, exposure to air, asphyxia postmortem change', etc. Rous also quotes Vlès as testing the reaction within frozen and ground mouse tissues by various physico-chemical methods and finding a hydrogen ion concentration in the mean of 6.0, which is nearer to the figure of Rous than that of other workers using different procedures.

The findings of Rous and Vlès are in reasonably good accord with the membrane equilibrium calculations for the muscle in vivo.

Much has been claimed for the carbon dioxide/bicarbonate method [e.g. Stella, 1929; Fenn, 1936; Hill & Kupalow, 1930; Hill, 1931] for determining muscle pH. It has been supposed that subjecting the washed sartorius to an external tension of carbon dioxide corresponding to the plasma value, determining the bound carbon dioxide and applying the Henderson-Hasselbalch equation, would give the pH of the muscle in vivo. Hill & Kupalow [1930] state that 'the application of the Henderson-Hasselbalch equation to Stella's [1929, p. 64] CO₂ dissociation curve of resting frog's muscle shows that to obtain a pH of 5.6 one would have to subject the tissue to a partial pressure of CO₂ of the order of two atmospheres' and again they write that the Rous figure of 5.6 as used by Fiske and Subbarow is 'obviously quite wrong'. It would now appear that Hill & Kupalow have been wrong, for they assumed not only

an internal pH of 5.9 with plasma pH of 7.6. This latter figure, as a mean value for frog's blood, was obtained by Barkan *et al.* [1921] using an electrometric method, and would also correspond to Fenn's figures of 25 m.equiv./l. for the plasma bicarbonate, and carbon dioxide tension of 20 mm. If the pH in vivo is 5.9 the change to 6.2 or 6.7 for the immersed muscle will necessitate the entrance or formation of alkali amounting to 5–14 m.equiv./kg. (for calculation see later) and the consideration of this entrance is essential for interpreting CO_2 and bicarbonate equilibria. It could be derived in the following ways.

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in concentration of HCO_3^- within the fibre is given by $(6.7 - \text{pH}) \times 27 \times 0.55$. We may write then

$$\text{pH} = 6.1 + \log \frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \quad (30)$$

$$\text{and} \quad [\text{HCO}_3^-] = (6.7 - \text{pH}) \times 14.9. \quad (31)$$

From these equations we may derive

$$[\text{HCO}_3^-] = 8.94 - 14.9 \log \frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]}. \quad (32)$$

From this equation on inserting any given value of the ratio $[\text{HCO}_3^-]/[\text{H}_2\text{CO}_3]$ we can solve for $[\text{HCO}_3^-]$ and hence for $[\text{H}_2\text{CO}_3]$ as m.equiv./l. fibre water. The $[\text{HCO}_3^-]$ figure multiplied by 122.67 gives the total present per litre fibre water with associated interspace which when multiplied again by 0.67 gives the $[\text{HCO}_3^-]$ as m.equiv. kg. muscle. This may be converted to vol. % by multiplying by 2.23. From Stella's figures when the carbon dioxide tension was zero a value for the bound carbon dioxide of 2.7 vol. % was obtained, and

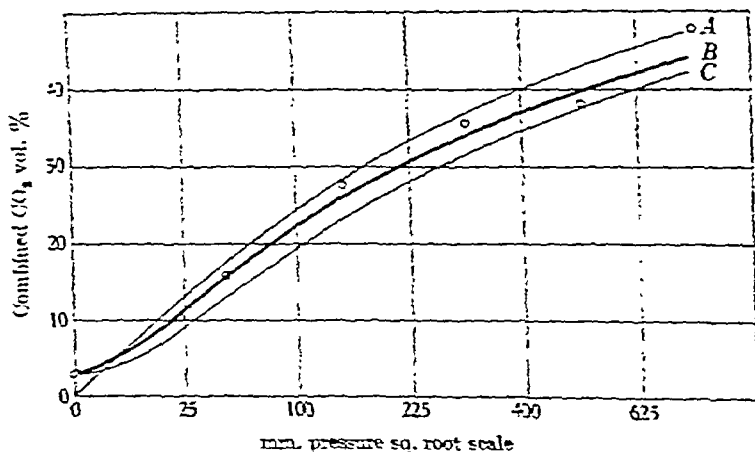


Fig. 14. The circles give the mean values of bicarbonate concentration (vol. bound carbon dioxide %) in washed sartorii subjected to varying carbon dioxide tensions, from Stella's data [1929]. The thick line (curve B) represents the theoretical curve as described in the text with a pK' for carbonic acid of 6.1. The lower thin curve (C) is for a pK' value of 6.2. The upper thin curve (A) is the empirical curve of Stella.

although this may be considered as zero within the sampling error, it seems preferable to regard it as a real quantity if only as corresponding to a blank value. This figure has been added to the calculated $[\text{HCO}_3^-]$ results. (The $[\text{H}_2\text{CO}_3]$ figures may be converted to mm. partial pressure by multiplying by $760/40.5$, where 40.5 represents the millimols carbonic acid dissolved in 1 l. of water under 760 mm. tension at 15°C .) From these $[\text{HCO}_3^-]$ and mm. CO_2 figures the curve B of Fig. 14 was made, where as in Stella's diagram the mm. partial pressure is plotted as the square root function to avoid too much crowding towards the origin when the full curve is drawn. All such curves are necessarily S-shaped at the beginning so that Stella's simple curve passing through the origin is incorrectly drawn, and conclusions drawn from the first part of the curve are liable to considerable relative error. Thus 12 vol. read off at 20 mm. may be 4 vol. too high.

The circles in Fig. 14 give the means of Stella's experimental data. What is remarkable about the theoretical curve B is that it expresses the mean experimental values even better than the empirical curve of

that potassium and anions were indiffusible across the membrane *in vivo*, but that they continued to be so in the excised and washed sartorius muscle. This they supposed to have, or to reform, the identical *in vivo* electrolyte content when under the plasma tension of 20 mm. carbon dioxide. As we have seen not only has sodium and chloride entered the fibres in very appreciable amount and potassium been lost, but the maintenance or stabilization concentration of potassium has also greatly risen, and that such stabilization represents a real equilibrium tendency may be judged from the prolonged immersions in the cold. One may say, in short, that the numerical data for the electrolyte balance across the membrane has radically changed; and the bicarbonate found is largely an expression of the effective alkali that has entered after excision and during the washing period.

What we regard as the true significance of Stella's data [1929] and the exact buffering of excised and washed muscle may now be dealt with in the light of the above considerations.

From our previous considerations the pH after washing may be taken as 6.7 within the fibre, with diffusible anion ratio of 1 : 4.2, and effective interspace volume of 13 ml./100 g. The pK' value for carbonic acid in human plasma from the data of Cullen, Keeler & Robinson [1925] is 6.19 at 18° C. The ionic strength in the 'free water' of the muscle fibre of the frog can be taken as even greater than in human plasma, so that the pK' value will be somewhat less than 6.19. The figure therefore may be regarded as lying between 6.1 and 6.2 and we have used both figures in subsequent calculations while considering 6.1 as the more probable.

It is true that Meyerhof, Möhle & Schulz [1932] have considered the pK' value in muscle to be 6.40 or 0.14 greater than the value for bicarbonate-Ringer solution at 20° given by Warburg [1922]. Their estimate of this figure rests, however, on a fallacy. They assume that two solutions both containing bicarbonate and the same tension of carbon dioxide but one containing also phosphate (or phosphate plus serum) have necessarily the same pH value if there is no change of carbon dioxide tension on mixing. Why this assumption was made is difficult to understand, but presumably they were influenced by the consideration that under such conditions there could be no change in total bicarbonate as there was no change in carbon dioxide, and hence no alteration of the hydrogen ion due to this system alone; but, marked changes can be accommodated by the other system involved, as the activity of their ions and of the bicarbonate ion alter with the ionic strength of the solution.

It is sufficient to note here that at least the *direction* of the various effects described by Meyerhof *et al.* can be deduced without difficulty from the current theory of activities and ionic strengths.

When the muscle as above is subjected to a carbon dioxide tension the pH falls and HCO_3^- is formed within. Potassium bicarbonate will then diffuse into the interspaces until the product of the potassium and bicarbonate ions is the same without as within and this may be expected to occur rather rapidly owing to the large surface and small volume of the fluid affected. The total HCO_3^- formed will then be distributed between the fibre water and the interspaces in the ratio of 67 to 13×4.2 , or $67/122$ (≈ 0.55) of the whole is present in the fibre water (4.2 being the ratio of concentrations across the membrane as considered above for chloride). The buffering power of muscle is 27 m.equiv./l. fibre water or 18 m.equiv./kg. muscle (from the data in § IV; vide Table 3) so that the increase

the non-protein buffers together have a practically linear effect over the wide range of pH from 5.5 to 7.1 may be deduced from the data in Table 3 and the usual buffering equation for weak acids or bases and that the muscle protein in turn with its multiple dissociating radicles is also mainly linear over such a range may also be assumed. Conversely, Stella's data in themselves give the exact muscle buffering and show its linear relation to the pH as in Fig. 15. In this the total $[HCO_3^-]$ figures have been plotted against the pH as determined from the equations above. Here any possible alternative pH values will merely run parallel with those plotted and the total $[HCO_3^-]$ figures represent experimental data. The linearity is clearly shown, and the magnitude of the buffering (27.4 m.equiv./l. 'fibre water') is identical with that deduced from the known analytical data for the phosphated compounds and carnosine and the analogy of the protein buffering with serum albumin and globulin as already considered.

Concluding on this question of the pH in the sartorius muscle fibre, it may be said that the other evidence, when it is or can be validly taken, agrees as we might expect with the membrane equilibrium deductions, the pH in vivo being approximately 5.9, changing to 6.7 on excision and immersion in ordinary Ringer solution, but apparently not exceeding 6.2 when the immersion is carried out in the cold. When the washed sartorius muscle is subjected to carbon dioxide tensions of 20 mm. its pH is 6.6 and not 6.9 as given by Stella, the latter figure being much in error when applied to the muscle in vivo. We may now refer to the work of Chambers *et al.* [1927] in determining the pH of cells by injecting dyes directly into them. These have been cited by Hill and others as supporting the conclusions of Stella & Fenn. Why it should be thought that dyes so injected should give better results than when they diffuse into the cells of the intact animal we are at a loss to understand, but seeing that even the excision of the sartorius muscle and its immersion in Ringer solution induces considerable alterations in the numerical data of electrolytes across the membrane, it can scarcely be supposed that the actual piercing of the membrane of a cell and the associated disturbances leaves it quite unaffected.

The bicarbonate, lactate and phosphate concentrations of muscle

The bicarbonate content of the muscle fibres has been stated on the basis of such experiments as those of Stella & Fenn to be 12 vol. %. It has been shown in the preceding section that such figures are illusory and merely represent a portion of the effective alkali that has entered

Stella which was drawn merely as a line of best fit. Curve *C* in Fig. 14 gives the theoretical curve when the pK' value of H_2CO_3 is taken as 6.2. It runs parallel with the experimental data and shows that if we were to take this value of the pK' it would be necessary at the same time to suppose a higher 'blank' value with zero carbon dioxide tension.

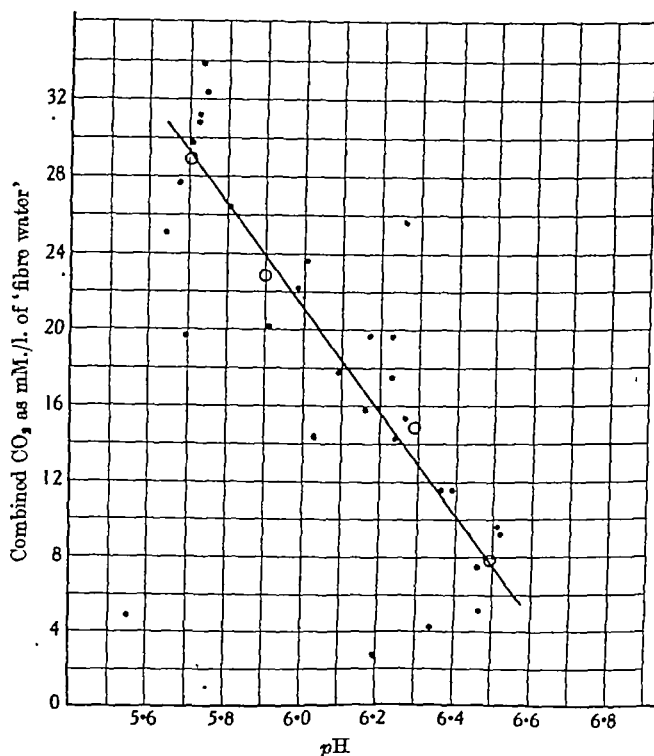


Fig. 15. The dots represent (from Stella's data, 1929) the total bicarbonate formation in washed sartorii subjected to varying carbon dioxide tensions (the small value for zero tension being subtracted) and expressed as mM./l. of 'fibre water'. The corresponding pH values are derived as described in the text. The large circles give mean values and the line is one of best fit. From this line we may derive the buffering of sartorii washed in bicarbonate free Ringer fluid (containing 8 mg. % P at 7.3 pH) to be 27.4 m.equiv./l. 'fibre water' or $27.4 \times 0.67 = 18.3$ per kg. muscle. It is evident that this buffering is practically constant over the significant physiological range of pH.

In the construction of the theoretical curve, we assumed that the buffering of the muscle was linear over the pH region covered. This may appear open to question. As already considered the buffering is made up of that given by the various phosphate esters and carnosine plus protein buffering, the protein constituting about 60 % of the total. That

in vivo as shown by the work of Hahn *et al.* [1939]. In the natural growth of the cell therefore, when the phosphate complex anions accumulate inside they must do so subsequent to the entry of diffusible phosphate, which is changed to indiffusible anion. An equivalent amount of potassium enters with the phosphate and is held when this latter becomes indiffusible, the equilibrium concentration of the free inorganic phosphate anion being quickly restored to the original level. The internal increase of ions causes an entrance of water and thus of cell volume. As the cell grows therefore potassium is accumulated *pari passu* with the accumulation of the organic esters and this process is one inevitably occurring with the kind of membrane we know to exist in muscle.

The phosphated esters, however, do not account for the whole of the potassium (and in such calculations we must include the internal indiffusible bases) but appear to be associated at least with the major part. An appreciable fraction of the potassium cation is balanced by negatively charged protein. For animals with digestive and circulatory systems we may consider this protein as built up from amino acids coming from the blood and that the total negative charge on the protein amounts to the total balance of negatively charged amino acid ions which have crossed the membrane. We may, therefore, as a net result consider the process as similar to that concerning phosphate, diffusible anions being changed to indiffusible with corresponding entrance and accumulation of potassium within the cell.

With this view potassium must accumulate automatically with the natural growth of the cell, no special mechanism being required, apart from the membrane and its characteristic permeability.

Where the cell receives its nitrogen from the simplest nitrogenous substances such as ammonia, it is obvious that the above considerations do not apply for that fraction of the potassium associated with protein. Here the cell must be regarded as forming its own excess of organic anion and necessarily with a corresponding amount of hydrogen ion. As a secondary process the hydrogen ion will interchange for potassium and this will then accumulate to the full extent of the organic acid formed; but it is to be noted that organic acids of the type of phosphocreatine, hexosemonophosphoric acid or adenylypyrophosphoric acid cannot possibly be formed *de novo* within the cell, but must arise from the interaction of the phosphate anion already associated with inorganic cations.

When by such a process as that outlined potassium accumulates in cells, the membrane may become subsequently cation impermeable. If with this there is loss of anions previously indiffusible, which will be

the excised and washed muscle. The bicarbonate content of the fibres from the membrane equilibrium views above should be given by $2.5/126$ (the inverse of the potassium ratio) multiplied by the plasma value, or 25 m.equiv./l. This gives 0.5 m.equiv./l. fibre water or 0.33 per kg. muscle (excluding the interspace bicarbonate, which is 0.13×25 or 3.25 m.equiv./kg.). In vol. % the bicarbonate within the fibres of 100 g. muscle may be taken as 0.7 and the total, including interspace bicarbonate, as 8.4 vol. %.

(The slightly different bicarbonate figure in Table 2 arises from a different calculation and the approximation of 0.13 for 0.127 as interspace volume.)

Concerning the very labile character of lactate and phosphate quantities in muscle and, for example, the inaccuracy inherent in such muscle phosphate determinations as those of Stella [1928], no value short of a general rough indication of the order of magnitude can be assigned to such determinations. We know on the other hand that when these anions are much increased in an external Ringer fluid that their concentrations in the muscle are relatively very similar to chloride, the lactate being practically identical [Ghaffar, 1935] and the phosphate somewhat lower [Eggleton, 1933] as may be expected.

A general biological view of the mechanism of potassium accumulation in cells

In the results described above it was shown that fresh excised muscle, owing to certain peculiarities of its membrane permeability to ions, could concentrate potassium against a gradient to a remarkable extent. This could happen without volume change when the conditions were correctly but very simply adjusted. Sodium was practically excluded by the membrane, and strictly so in the cold with raised potassium; also the indiffusible anions within the membrane did not appreciably decrease. It would be strange indeed if this mechanism of accumulation had no bearing on that naturally occurring in the growth of cells. We are emboldened to extend it to a consideration of the original entry of potassium, but can only indicate briefly the further principles involved.

Potassium in muscle (and it would seem generally in cells) may be considered as held by the electrostatic attraction of the indiffusible anions. An osmotic balance occurs also as we have seen and the cell maintains a definite equilibrium volume. The indiffusible anions which hold the electrical balance with potassium are mostly esters of organic acids and phosphoric acid. The inorganic phosphate anions are diffusible

outlined above, and then the secretion of potassium chloride therefrom with constant energy expenditure in a way not yet adequately explained.

According to the explanation advanced by Osterhout *et al.* potassium hydroxide passes through an external non-aqueous layer or membrane and is then neutralized by organic acids which subsequently exchange for chloride without. This view, at least in its present form, would appear open to the following criticism:

(a) Since a non-aqueous layer or membrane is assumed, ionic penetration would seem largely outruled, and in favour of this Osterhout *et al.* bring forward supporting evidence, though holding again, and it would appear somewhat inconsistently, to a possible exchange between chloride and the organic acid anion. Considering the direct acidic exchange it would seem impossible that hydrochloric acid could diffuse across a passive non-aqueous layer against a thermodynamic gradient, since the concentration of the acid in the internal edge of the layer would exceed that in the outer. In a mono-molecular layer this may need to be qualified by other considerations, which, however, have not been advanced. It is not surprising therefore that the model, as considered by Osterhout & Stanley [1932] and Osterhout & Kamerling [1935], fails to show a movement of chloride across a non-aqueous layer in the direction of the potassium accumulation (carbon dioxide being used as the organic acid and 70 % guaiacol and 30 % *p*-cresol as the non-aqueous layer). On the contrary, the chloride movement (apart from changes in the thermodynamic potential of potassium chloride) is in the direction of the thermodynamic gradient. The model fails therefore to represent an essential feature of the accumulation of potassium chloride in the cell sap of *Valonia*.

(b) The apparent energy waste in the supposed exchange of an organic acid for hydrochloric acid in *Valonia* is very high. Taking carbonic acid as presumably the most economical acid for this exchange, it would need to be produced at the rate of about 17,000 c.c. carbon dioxide per kg. of protoplasm per hour, needing, we may suppose, a somewhat similar oxygen intake. This calculation for *Valonia*—assuming free CO_2 to escape at least as fast as HCO_3^- —is based on the following figures:

Protoplasmic layer	A few μ thick
Volume of typical cell	1 c.c.
KCl in sap	580 m.equiv./kg.
Growth per day	1 % of the whole
pH of sap	5.8

largely replaced by chloride, the potassium and chloride product will be greater inside the membrane. We should have in short the conditions seen in the matured red corpuscle and possibly considerations of a similar kind may be extended to nerve tissue, for it would appear from the findings of Webb & Young [1940] for the single giant fibres in the nerves of the squid (*Loligo forbesi*; similar to Bear & Schmitt [1939] for the *Loligo pealii*) the potassium and chloride products inside the membrane exceed those outside.

This view of potassium accumulation appears to us as being probably of general application to cells, but we put it forward also in the belief that deviations therefrom may well occur in the wide diversity of cellular adjustment to environment.

Other views on the biological accumulation of potassium

Here we may confine ourselves to a consideration, necessarily brief, of the views of Osterhout *et al.* [1931-5] concerning the accumulation of potassium in the cell sap of the *Valonia macrophysia*, their explanation of the process differing from that given above. The nature of the problem is, however, very dissimilar. The *V. macrophysia* is a one-celled marine plant which has an external pellicle of protoplasm a few μ thick inclosing a comparatively very large amount of cell sap, so that the total volume of the cell may reach the size of a pigeon's egg. The potassium in the cell sap is about 42 times more concentrated than in the surrounding sea water, whereas the sodium concentration is only 0.18 of the external value. At the same time the chloride content of the sap is only very slightly greater than that of the external chloride (1.03 times). Clearly there is here no question of an equilibrium relation of potassium chloride within and without, the ionic products being very different, but rather the establishment of a steady state of concentration with energy expenditure as the cell grows. Consequently, when the temperature is lowered there is a loss of potassium from the cell sap and a gain of sodium. This is very different from what happens with animal tissue cells, a fall of temperature having no appreciable effect on the potassium ratio. The problem therefore of potassium concentration in *Valonia* cell sap, though apparently a comparatively simple one for the study of potassium accumulation, is complicated by the fact that the formation of the cell sap from the pellicle of protoplasm must be likened to an active secretion such as occurs in certain cells of higher organisms. We should prefer to regard the total process as occurring in two stages, firstly the entrance of potassium and chloride into the pellicle of protoplasm in the manner

millimols), and of ϵ the difference between the total negative and positive charges on the indiffusible molecules (expressed as milliequivalents or valencies multiplied by the millimolar concentrations).

3. Changes in η and ϵ produced by wide changes in potassium concentration are shown to be relatively insignificant both theoretically and experimentally. It happens that the mean value of η is practically the same as ϵ for the immersed sartorius, which leads to a more useful equation for the volume change.

4. Expressing the concentrations of potassium and chloride in the 'fibre water' in accordance with the findings of the previous paper, it is shown that the mean experimental results are in very good accord with the theoretical requirements. The Donnan relation applies very satisfactorily for a wide range of external potassium concentration (12-300 m.equiv./l.).

5. It is shown both theoretically and experimentally that the volume of immersed sartorii remains practically constant despite the largest additions of potassium chloride to the external solution, provided only that the sodium concentration is maintained constant. If, on the other hand, the total external concentration is maintained unchanged but potassium substituted progressively for sodium in equivalent relation, the muscle volume increases to equilibrium values reached by 24 hr. immersion in the cold. The theoretical equation given describes quantitatively the mean equilibrium volume up to 100 % increase of the 'fibre water' (or to about 70 % of the total muscle weight).

6. The volume of water in which the normal or the accumulated potassium in muscle is dissolved—the 'osmotically active water'—is shown to be 0.67 time the original muscle weight. This corresponds to the total water minus the interspace water.

7. Resting potentials in the sartorii from curarized frogs are described by the theoretical equations, and are fully explained by potassium equilibria across the membrane.

8. Gains of sodium and of chloride, and losses of potassium, have been studied for sartorii immersed for 2 hr. at room temperature in ordinary Ringer-Locke, Ringer-Barkan and a special solution, the latter resembling the electrolyte content of frog's plasma as closely as possible from the more recent analyses.

9. The maintenance concentration of potassium or that required to prevent loss from immersed muscle has been found to be 29 m.equiv./l. for 2 hr. at room temperature (using Ringer-Barkan fluid) and 10 m.equiv./l. for 24 hr. immersion in the cold. The significance of these

(To these figures may be added a pK' value of 6.1 for carbonic acid in sea water or *Valonia* cell sap.) The neutralization of the entering potassium hydroxide is considered to occur at the pH of 5.8 which is steadily maintained within the sap.

Considering the above figure of 17,000 c.c. of carbon dioxide, even the mouse requires only 4900 c.c. oxygen/kg./hr. corresponding to about 1000 c.c. at 20° C. (by comparison with the effect of temperature on the oxygen consumption of the rabbit [Conway, O'Connor & Donovan, 1937]. The highest figure we could find for invertebrate consumption was 1400 c.c. oxygen/kg./hr. for the insect *Bombyx Mori* [Batelli & Stern, 1913] and the energy requirement of bacteria is in the mean [Rubner, 1906, 1909] only 3-4 times that of the mouse.

The neutralization of the KOH by formic acid would seem a somewhat more promising possibility considering quantities only, but higher acids may be reasonably excluded.

We may conclude by emphasizing that, judging from our results with muscle, the study of potassium accumulation in cell sap with continuous energy expenditure is a quite different and more complicated study than the accumulation within the substance of cells, involving in itself no continual energy expenditure, but relating to the establishment of equilibria. These equilibria in turn are largely conditioned by the nature of the synthetic products formed by the cell, especially the phosphate esters, and by the characteristic permeabilities of the membrane.

SUMMARY

1. The study of potassium, sodium and chloride changes in sartorii immersed at 2-3° C. in modified Ringer solutions shows that potassium, as the chloride, can be accumulated to upwards of three times the normal concentration. This can occur without volume change, provided the solution is suitably adjusted. With raised potassium concentration, sodium is practically perfectly excluded for over 24 hr. There is only a negligible loss of previously indiffusible molecules, which for the most part consist of phosphate esters. The muscle membrane is therefore permeable both to cations and anions up to certain size limits.

2. The theoretical development of such a membrane system, based on a consideration of the osmotic, electrical and Donnan equilibria results in equations which predict the muscle volume, the internal potassium and diffusible anion concentration, the resting currents and the currents of injury, in terms of the external concentrations, and the quantities η and ϵ . The value of η represents the total indiffusible substance (as

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relatively high maintenance concentrations for excised muscle is discussed, and the explanation given accounts *quantitatively* for the sudden entrance of chloride into sartorii immersed in Ringer solution.

10. The permeability of resting muscle *in vivo* is considered in the light of the recent findings with radio salts.

11. The question of the pH and bicarbonate content of the fibres of resting muscle is dealt with, and the bicarbonate and carbon dioxide data of Stella [1929] are exactly interpreted from the new standpoint. The error involved in applying such results directly to muscle *in vivo* is indicated.

12. It is shown that the total buffering of excised muscle previously washed in bicarbonate free Ringer solution is linear over the physiologically significant range of pH, and is equal to 27.4 m.equiv./l. of 'fibre water' or 18.3 per kg. muscle. This buffering can be exactly accounted for by the phosphate esters, carnosine and the muscle protein when this latter is considered to have a buffering value between serum globulin and serum albumin.

13. A general view of the manner in which potassium is concentrated biologically is put forward, and certain criticisms made of the views of Osterhout *et al.* with respect to the concentrating of potassium in the cell sap of the *Valonia macrophysia*.

Our thanks are due to Prof. F. G. Donnan for the very friendly interest he has taken throughout this research, as well as for some criticism. One of us is also indebted to the Medical Research Council of Ireland for a personal grant.

The theoretical plan of the research is due to the senior author.

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interesting property of antagonizing, or in some instances reversing, certain actions of adrenaline, and to this particular effect I have previously made brief reference [Gunn, 1936].

TOXICITY

The supply of the compound available was not sufficient to permit toxicity experiments on large animals. The lethality was determined by intraperitoneal injections in white mice. The approximate M.L.D. was first estimated by the effects of graded doses on single animals, and subsequently the L.D.₅₀ determined on three groups, each of ten mice. The L.D.₅₀ was found to be 0.11 g./kg.

The L.D.₅₀ for frogs (*R. temporaria*) by injection into the dorsal lymph sac was similarly estimated and found to be approximately 0.25 g./kg.

SYMPTOMS

Mice. The characteristic symptoms seen when a mouse received intraperitoneally a lethal dose of the alkaloid were as follows:

After one or two minutes, the animal began to move hesitantly round the cage and presently showed signs of increased nervous excitability, especially by jerky movements of the head and neck and by champing of the jaws. Usually following a squeal, the mouse made a rapid rush which ended in an epileptiform convulsion during which the animal might leap in the air. Respirations became rapid and shallow, but convulsions occurred before there was any serious depression of respiration and before there were any signs of cyanosis. Similar convulsions occurred at short intervals, but they became less violent and later the animal remained prone with the hind limbs splayed out. During this stage rotational convulsive movements round the long axis of the body were frequently seen. Death occurred either from respiratory failure immediately following a convulsion, or from gradual enfeeblement of respiration accompanying general motor paralysis. When the chest was opened immediately after arrest of respiration, the heart was found to be beating feebly and it stopped finally in the diastolic position.

With doses of 0.3–0.1 g./kg. death occurred in from 5 to 30 min. after intraperitoneal injection. Doses less than 0.05 g./kg. caused only slight symptoms, mainly tremor and ataxia. With non-fatal doses, recovery was complete in a few hours and surviving animals showed no symptoms of delayed poisoning.

Frogs. In frogs this compound produced gradual motor paralysis without any symptoms of previous excitation. When respirations stopped,

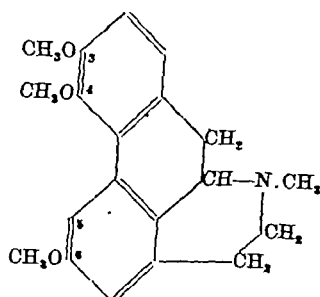
THE PHARMACOLOGICAL ACTIONS OF MORPHOTHEBAINE-DIMETHYLETHER

By J. A. GUNN

From the Nuffield Institute for Medical Research, Oxford

(Received 16 December 1940)

At intervals during the past few years I have investigated the actions of the dimethylether of morphothebaine, a compound which I received in the form of the dextro-bitartrate from Prof. J. M. Gulland. The synthesis of the compound was described by Gulland & Haworth [1928]. It has the formula described below:



It belongs to the aporphine group of alkaloids, being 3, 4, 6-trimethoxy-aporphine. Many related alkaloids are known which have hydroxyl or methoxyl groups in the 3, 4, 5, 6 positions. Apomorphine is 3, 4-dihydroxy-aporphine, but perhaps the most closely related alkaloids are bulbocapnine (3-methoxy, 4-hydroxy, 5, 6-methylenedioxy, aporphine) and corydine (3, 4, 6-trimethoxy, 5-hydroxy, aporphine). The latter differs from morphothebaine-dimethylether only in possessing a hydroxyl group in the 5 position.

It is hoped to make a comparison of the pharmacological actions of some of these related alkaloids at a later date.

The actions of this particular compound have not previously been described. In the course of the investigation it was found to have the

action of adrenaline. The most common effect is seen in Fig. 2. A dose of 8 μ g. adrenaline raised the blood pressure by 55 mm. Hg. Morphothebaine-dimethylether was then injected in a dose of 0.024 g./kg. This

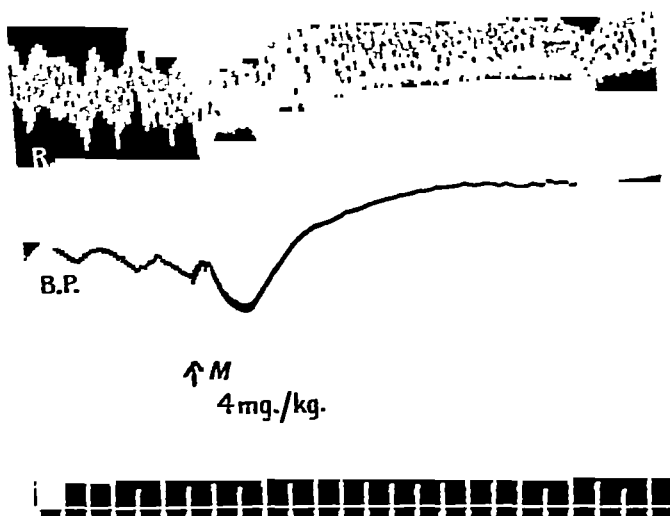


Fig. 1. Rabbit, urethane anaesthesia. Record of respiratory movements and blood pressure, showing increased rate of respiration and fall, followed by rise, of blood pressure by M.T.E., 4 mg. kg. Initial B.P. = 80 mm. Hg; a vertical movement of 8 mm. is equal to a change of 10 mm. in the original tracing.

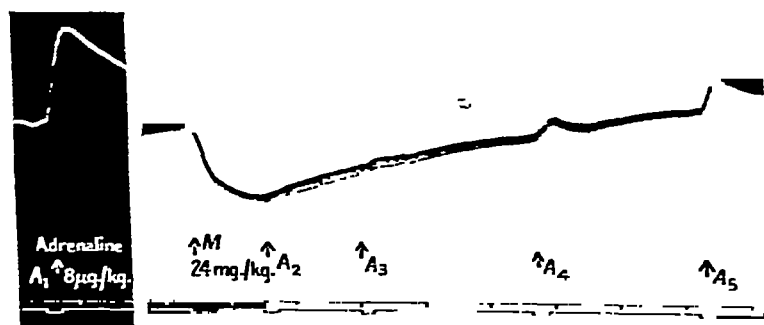


Fig. 2. Rabbit, urethane anaesthesia, record of blood pressure, showing rise of blood pressure by adrenaline (A_1), fall of blood pressure by M.T.E. (M) and subsequent abolition, with gradual recovery, of the pressor effect of the previous dose of adrenaline (A_{2-5}). Initial B.P. = 100 mm. Hg; a vertical movement of 5 mm. is equal to a change of 10 mm. in the original tracing.

produced a fall of blood pressure of about 40 mm. Hg. During the gradual recovery of blood pressure, successive injections of the previous dose of adrenaline were given at intervals (A_{2-5}) with the following results

the circulation (as observed in the web of the foot) was still fairly vigorous. The heart continued to beat for some time after arrest of respiration. When the chest was opened immediately after arrest of the heart, both auricles and ventricle were found to be in the diastolic position. At the time of motor paralysis and even after arrest of the heart, stimulation of the sciatic nerve with a weak faradic stimulus still caused vigorous contraction of the gastrocnemius muscle, indicating that the motor paralysis was central and not peripheral.

ACTION ON THE CENTRAL NERVOUS SYSTEM

No special experiments were made to determine the exact site and nature of action of the compound on the nervous system, but a rough idea of the actions can be gathered from the general symptoms. In mice, the increase of voluntary movement culminating in clonic convulsive movements, with the absence of any symptoms suggestive of increased spinal excitability, point to a supra-spinal stimulation of the central nervous system. The absence of increased excitability of the central nervous system in frogs supports this view. The primary stimulation in mice may be followed by depression of the central nervous system and the latter is the only obvious effect in frogs.

BLOOD PRESSURE AND RESPIRATION

Rabbits. The rabbits were anaesthetized by urethane, 2 g./kg. subcutaneously.

Smaller doses of morphothebaine-dimethylether (M.T.E.) (0.001-0.01 g./kg. intravenously) caused a fall of blood pressure with stimulation of the respiratory movements. For example, in one experiment a dose of 0.002 g./kg. intravenously lowered the blood pressure from 80 mm. Hg to a minimum of 50 mm. Hg and increased the respiration rate from 60 to 90 per min.

Doses below 0.005 g./kg. sometimes produced a slight rise of pressure above the normal, following the preliminary fall. This two-fold effect on blood pressure with respiratory stimulation is illustrated in Fig. 1. In this case, after a dose of 4 mg./kg., there was a transient fall of blood pressure from 80 to 60 mm., followed by a more sustained rise to a maximum of 100 mm. The respiration rate increased from 42 to a maximum of 60 per min. Larger doses (above 0.01 g./kg.) produced a more profound and sustained fall of blood pressure with slowing of the respiration rate.

The most interesting point about the action of this compound on blood pressure is that it can annul, or sometimes even reverse, thepressor

These and similar experiments showed that, in the rabbit anaesthetized by urethane, this compound can annul, or sometimes reverse, the pressor effect of adrenaline, but that this action is very transient. Even within a few minutes the pressor effect of adrenaline begins to re-establish itself.

TABLE 1

Time	Injection	Blood pressure	Heart rate in 10 sec.
11.29		64	15
11.30	Adrenaline 2 μ g./kg.		
11.30 $\frac{1}{2}$		105	22
11.36		82	14
11.37	M.T.E. 10 mg./kg.		
11.37 $\frac{1}{2}$		54	13
11.38	Adrenaline 2 μ g./kg.		
11.38 $\frac{1}{2}$		74	16
11.49 $\frac{1}{2}$		62	11
11.50	Adrenaline 2 μ g./kg.		
11.50 $\frac{1}{2}$		110	21
12.0	M.T.E. 20 mg./kg.	74	11
12.0 $\frac{1}{2}$		50	10
12.1	Adrenaline 2 μ g./kg.		
12.1 $\frac{1}{2}$		58	14
12.2		62	14
12.2 $\frac{1}{2}$	Adrenaline 2 μ g./kg.		
12.3		94	18

Cat. The experiments were made on spinal cats. The effects of M.T.E. on blood pressure were very similar to those described in rabbits. With

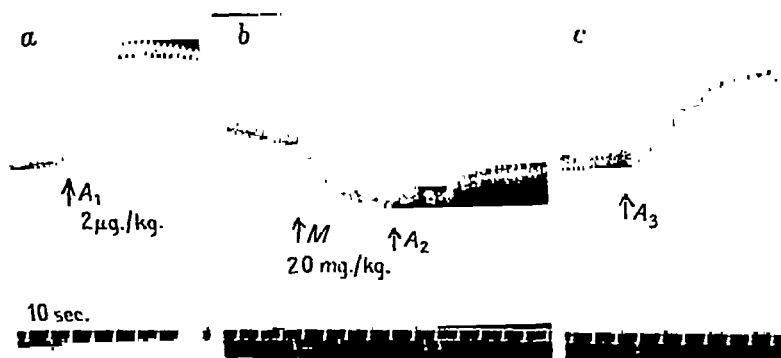


Fig. 4. Spinal cat, blood pressure showing normal pressor action of adrenaline (A_1), depressor effect of M.T.E. (M) immediately following which the pressor effect of adrenaline is almost annulled (A_2), but is nearly completely restored within 2 min. (A_3). Initial B.P. = 64 mm. Hg; a vertical movement of 7 mm. is equal to a change of 10 mm. in the original tracing.

the slower heart rate in cats it was possible to determine the effects on heart rate as well as on blood pressure. The following experiment (Table 1 and Fig. 4) illustrates the results obtained.

(seconds after beginning of injection: pressor effect of adrenaline in mm. Hg): 15 sec., 0; 30 sec., 2; 60 sec., 10; 90 sec., 18. This experiment is typical of a result which has been obtained many times and which shows that, after injection of a sufficient dose of this compound, the pressor effect of adrenaline is transiently annulled but is gradually re-established after a few minutes.

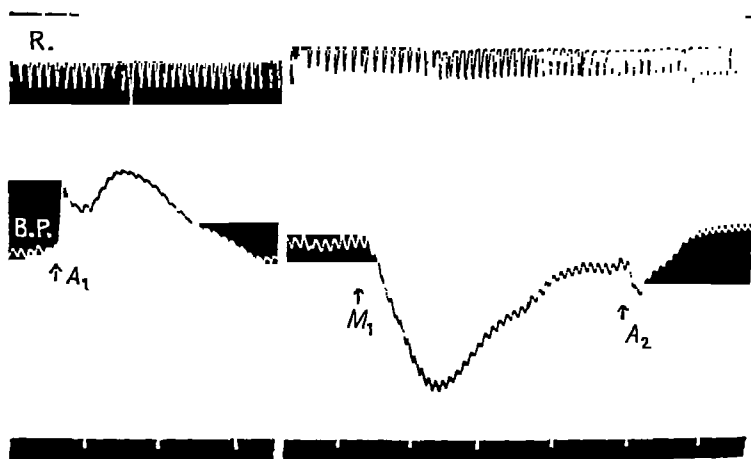


Fig. 3. Rabbit, urethane anaesthesia, record of respiratory movements (R.) and blood pressure (B.P.), showing increased rate of respirations and fall of blood pressure by M.T.E. 20 mg./kg. at M_1 , and subsequent reversal of pressor action of adrenaline, the same dose of adrenaline, 10 μ g./kg., being given at A_1 and A_2 . Initial B.P. = 120 mm. Hg; a vertical movement of 4 mm. is equal to a change of 10 mm. in the original tracing.

Less commonly there is a brief period after injection of this compound during which a slight reversal of the effect of adrenaline is obtained, i.e. a dose of adrenaline which previously produced a rise of pressure, caused, after injection of morphothebaine-dimethylether, a slight fall of pressure. Such a result is illustrated in Fig. 3. In this experiment an injection of adrenaline 10 μ g./kg. caused a rise of blood pressure of about 50 mm. Hg (A_1). When the blood pressure had returned to normal, morphothebaine-dimethylether was injected in a dose of 20 mg./kg. (M_1). This caused a fall of blood pressure to the extent of 90 mm. Hg and an increase in respiration rate from 8 to 11 per 10 sec. When the blood pressure had almost reached its normal level (about 40 sec. after injection of M.T.E.), the same dose of adrenaline caused an evanescent fall of pressure of about 15 mm. Hg (A_2). Two minutes later the same dose of adrenaline caused a rise of pressure of 10 mm. Hg.

pression caused by M.T.E., adrenaline failed to produce either augmentation or acceleration. Those effects are illustrated in Fig. 5. Tracing *a* shows the augmentor effect of adrenaline (1 in 1,000,000). The adrenaline solution was replaced by Ringer's solution, and later M.T.E. 1 in 50,000 was perfused through the heart (*b*). This at first diminished the excursions and later the rate of beat. Adrenaline was then added to the perfusing solution to give a concentration of 1 in 1,000,000, but this failed to produce any augmentation or acceleration (*c*, *A*), though the heart rapidly recovered when Ringer's solution was substituted (*c*, *RS*).

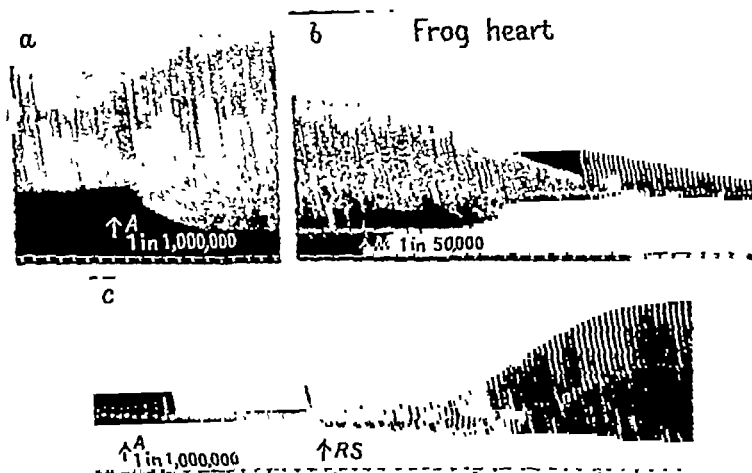


Fig. 5. Perfused isolated heart of frog, systole recorded by upstroke, showing (a) augmentor effect of adrenaline (1 in 1,000,000) at *A*, (b) depressant effect of M.T.E. (1 in 50,000) at *M*, during which (c) adrenaline fails to show any sympathomimetic effect at *A*, though the heart quickly recovers when reperfused with pure Locke's solution at *RS*.

Rabbit. The action of M.T.E. on perfused heart of the rabbit was in all respects similar to that on the frog's heart. Strong solutions, 1 in 50,000 to 1 in 20,000, caused at first a diminution in the force of contraction without alteration of the rate of beat; later the rate also was reduced, often with a temporary coincident improvement in the force of beat. The heart was finally arrested in a position of almost complete diastolic relaxation. In the heart of the rabbit as in that of the frog, M.T.E., if perfused in a concentration sufficient to produce marked depression of the muscle, prevented the stimulating effect of adrenaline. In parallel experiments with ergotoxine, I have never been able to annul the action of adrenaline, on the perfused hearts of either rabbits or frogs, in the same way as can be done with M.T.E.

First, in regard to the action of M.T.E. itself, a dose of 10 mg./kg. lowered the blood pressure from 82 to 54 mm., and the heart rate from 14 to 13 per 10 sec.; a dose of 20 mg./kg. lowered the blood pressure from 74 to 50 mm. and the heart rate from 11 to 10 per 10 sec. As reduction in the heart rate was so slight and as there was no coincident diminution of pulse volume, the evidence, so far as it goes, suggests that, with these doses, the fall in blood pressure is due mainly to dilatation of the vessels. Such a dilatation would be in keeping with the depressant effect which M.T.E. displays on other forms of smooth muscle.

Secondly, in regard to the effect of M.T.E. upon the pressor action of adrenaline, the experiment shows the following points of interest. Immediately after the injections of M.T.E. the pressor effect of adrenaline is much reduced. For example, before M.T.E. adrenaline (the same dose of it, 2 μ g./kg. was given at each injection) raised the blood pressure by 41 mm. and increased the heart rate by 7 beats per 10 sec., while after the injection of M.T.E. 10 mg./kg. adrenaline raised the blood pressure by 20 mm. and increased the heart rate by 3 beats per 10 sec. Both the pressor and cardio-accelerator effects of adrenaline were much reduced. The speed with which this 'sympatholytic' action of M.T.E. passes off is seen from the fact that, whereas 1 min. after the second injection of M.T.E. 20 mg./kg. adrenaline only raised the blood pressure by 8 mm. and increased the heart rate by 4 beats per 10 sec., 1½ min. later adrenaline raised the blood pressure by 32 mm. and increased the heart rate again by 4 beats per 10 sec.

It is evident from this that the effect of M.T.E. in preventing the manifestation of the pressor and cardio-accelerator actions of adrenaline is very evanescent, the normal quantitative action of adrenaline being almost re-established within a few minutes. The effects on blood pressure in the spinal cat are, therefore, similar to those found in the anaesthetized rabbit. In the former case, and by presumption in the latter, the depressor action of M.T.E. is independent of the vasomotor centre and is mainly due to peripheral relaxation of the blood vessels, though a contributory depressant action on heart muscle, especially with large doses, is not excluded, in view of the results obtained on the isolated heart.

ISOLATED HEART

Frog. The isolated perfused heart of the frog was arrested in complete or almost complete diastole by solutions of M.T.E. from 1 in 100,000 to 1 in 10,000. The initial effect was reduction in the force of systole without any change in rate of beat. Later the rate also was reduced. The effect was typical of a depressant action on cardiac muscle. During the de-

resumes its normal motor response to adrenaline if the M.T.E. is removed.

Experiments on the uterus in situ were done on rabbits anaesthetized by urethane (2 g./kg.). The animals were submerged up to the neck in a tank of normal saline kept at 37° C. The abdomen was opened in the mid-line and the uterus connected by a thread to a recording lever, upstroke indicating contraction. Under these conditions M.T.E., intravenously injected in any dose above 0.005 g./kg., caused a rapid and profound relaxation of the uterus with the abolition of spontaneous contractions if these were present. When the effect of M.T.E. on the motor

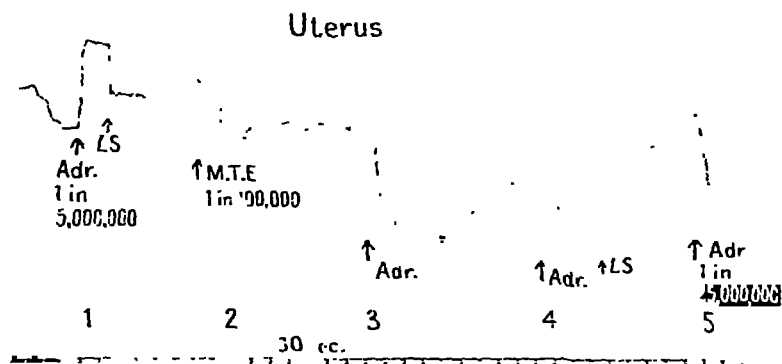


Fig. 7. Isolated uterus of rabbit, showing reversal of action of adrenaline by M.T.E.; the normal motor effect of adrenaline 1 in 5,000,000, (1) is, following M.T.E., 1 in 1,000,000 (2) replaced by a marked inhibitor effect (3, 4 and 5).

effect of adrenaline was investigated, it appeared in the first experiments as if the sympatholytic effect of M.T.E. on the uterus in situ was very feeble as compared with the corresponding effect on the excised uterus. As soon as it was realized that this effect was very transient and that, in order to demonstrate it, adrenaline had to be injected within a few minutes after M.T.E., then it was easy to show that, in the organ in situ as in the excised organ, M.T.E. diminishes or abolishes the motor effect of adrenaline on the rabbit's uterus. In only two out of six such experiments was anything in the nature of a reversal obtained, and in both those cases the relaxation produced by adrenaline after M.T.E. was very slight.

Most of these points can be illustrated by a single experiment (Fig. 8). In this experiment the dose of adrenaline was throughout 0.01 mg./kg.,

UTERUS

The isolated uterus of the cat and rabbit in all physiological states of the organ, is relaxed by M.T.E. Relaxation is pronounced with concentrations of 1 in 50,000 to 1 in 20,000 (vide Fig. 6) but is manifest even with a concentration of 1 in 250,000.

After subjection to a solution of 1 in 20,000 to 1 in 100,000 of M.T.E., the response of the rabbit's uterus to adrenaline is quantitatively, or sometimes qualitatively, altered. The uterus of the rabbit, with very rare exceptions, is powerfully stimulated by adrenaline. If a uterus showing this normal contraction response to adrenaline is subjected for a few minutes to the action of a solution of M.T.E. of a concentration not less than 1 in 100,000, the effect of the previous concentration of adrenaline may be either diminished, annulled or reversed. Reversal was shown best by uteri which were well developed but not pregnant.

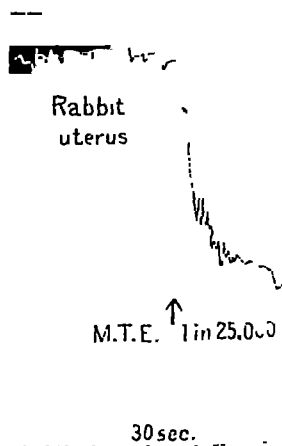


Fig. 6. Isolated uterus of rabbit, showing rapid relaxation of uterine muscle by M.T.E., 1 in 25,000.

An example of reversal of the action of adrenaline is shown in Fig. 7. In this experiment, adrenaline produced contraction of the uterus in the tested concentrations of 1 in 5,000,000 (Fig. 7, 1) and 1 in 25,000,000. A solution of M.T.E., 1 in 25,000, caused rapid and complete relaxation of the uterus (Fig. 6). When this solution was replaced by Locke's solution the uterus gradually recovered its tone.

A weaker solution of M.T.E. (1 in 100,000) then produced a slight relaxation (Fig. 7, 2). After 4 min. adrenaline, 1 in 5,000,000, produced a rapid and complete relaxation (Fig. 7, 3), an effect which was repeated 6 min. later (Fig. 7, 4). The drug solution was then replaced by Locke's solution and, 2 min. after this change, adrenaline, 1 in 25,000,000, still produced quite a considerable relaxation (Fig. 7; 5).

This experiment shows that M.T.E. can, in sufficient concentrations, produce reversal of the motor effect of adrenaline on the excised uterus and that this condition, once induced, can persist for several minutes, even after the M.T.E. solution has been replaced by Locke's solution. The condition is, however, easily reversible because in time the uterus

produced by any concentration of M.T.E. greater than 1 in 200,000. A concentration which produced a marked depressant effect on intestinal muscle did not, however, abolish the inhibitor effect of adrenaline. These points are illustrated in Fig. 9. A test concentration of adrenaline (1 in 10,000,000) produced relaxation of the intestinal muscle with diminution of the rhythmic movements (A_2). When the adrenaline solution was replaced by Locke's solution (L.S.) the rhythmic movements were quickly re-established. M.T.E. (1 in 50,000) was added at M , and

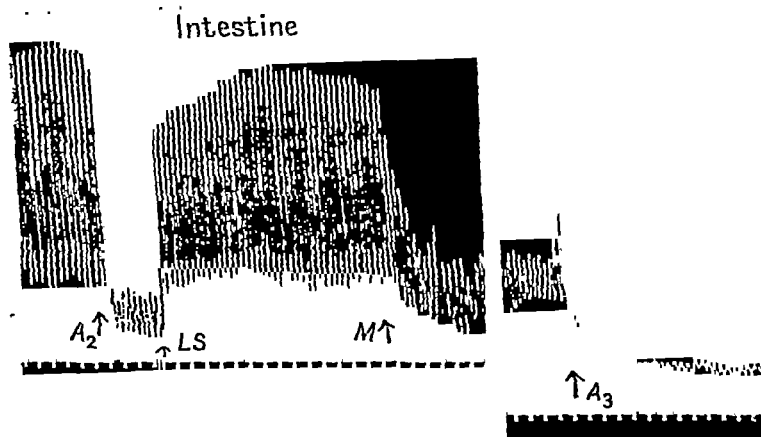


Fig. 9. Isolated intestine of rabbit, upstroke contraction; at A_2 and A_3 adrenaline 1 in 10,000,000; at M , M.T.E. 1 in 50,000; showing depressant effect of M.T.E. on intestinal muscle, but the inhibitor action (A_1) of adrenaline is not suppressed (A_3).

this also produced relaxation of the intestine with reduction of the rhythmic movements. While the intestine was in this condition adrenaline (1 in 10,000,000) still produced a typical further inhibitory effect.

SPLEEN

Experiments were made on the isolated spleen of the rabbit and cat. The muscle of the spleen, like that of the uterus and intestine, is relaxed by M.T.E. As in the case of the uterus, the motor effect of adrenaline on the spleen is diminished or abolished by M.T.E. Reversal of the motor effect of adrenaline was not obtained in any of six experiments. Fig. 10 shows first the motor effect of adrenaline 1 in 5,000,000 (A_1). At the height of the contraction M.T.E. was added to make a concentration of 1 in 50,000 (M). This produced a gradual relaxation of the splenic muscle.

and the successive intravenous injections given are mentioned in the order of the tracing. Before M.T.E., adrenaline produced a powerful contraction of the uterus, lasting about 4 min. (A_1). A dose of M.T.E. (0.01 g./kg.) then produced relaxation of the uterus with abolition of the spontaneous rhythmic contractions (M_1). Two minutes after the beginning of this injection, adrenaline produced a much feebler contraction lasting only about 20 sec. (A_2), but 6 min. later the motor effect of adrenaline was almost completely re-established (A_3). M.T.E. was later given in twice the previous dose (0.02 g./kg.), and this again produced relaxation of the uterus (M_2). After an interval of 80 sec. adrenaline

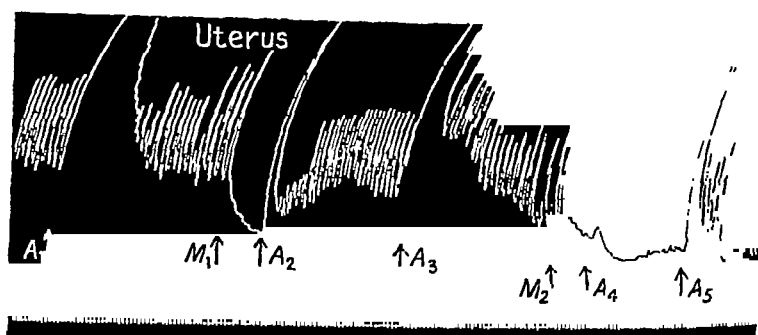


Fig. 8. Rabbit, urethane anaesthesia, record of uterine movements in situ; at A_1 , adrenaline 0.01 mg./kg.; at M_1 , M.T.E. 0.01 g./kg. and at M_2 M.T.E. 0.02 g./kg.; showing depressant effect of M.T.E. on uterine muscle following which the normal pressor effect (A_1) of adrenaline is reduced (A_2) or suppressed (A_4), but this effect is transitory (A_3) and (A_5).

was again injected and now had almost no effect on the uterus (A_4). After a further 4 min., however, adrenaline again produced a powerful contraction of the uterus lasting over 90 sec. (A_5).

From this and other similar experiments it was found that a sufficient dose of M.T.E. can reduce or abolish the motor effect on the rabbit's uterus in situ. The effect, however, is very transient, and the sympathicolytic effect, even of a dose which completely cuts out the motor action of adrenaline, may entirely disappear in 10 min.

INTESTINE

Experiments were made on the isolated intestine of the cat and rabbit. M.T.E. invariably caused relaxation of the intestinal muscle with diminution or suppression of the segmentation movements. This effect was

of action reduces the value of M.T.E. as a sympathicolytic agent for certain physiological purposes. As the effect on the isolated organ (e.g. Fig. 7) is more lasting than in the intact animal (e.g. Fig. 8), it is probable that M.T.E. is rapidly destroyed or excreted in the body.

So far as arterial pressure is concerned, reversal of action of adrenaline is obtained only uncertainly with M.T.E. Dale found reversal produced by ergotoxine readily in the cat, but in the rabbit there was usually 'a simple, progressive obliteration of the pressor effect'. Actually I have not obtained any reversal of the action of adrenaline after M.T.E. in the cat, the result being merely a suppression of the pressor effect of adrenaline, as in Fig. 4, while a slight reversal has sometimes occurred in the rabbit, as in Fig. 3.

Ergotoxine differs from M.T.E. in exerting a primary stimulant action on smooth muscle, whereas the latter depresses smooth muscle from the beginning. On the isolated heart, uterus, or spleen, for example, no stimulant action is seen even with the weakest effective concentration of M.T.E. After Dale [1906] had shown the 'adrenaline reversal' on the blood pressure of the cat, Cannon & Lyman [1913] suggested that the action of ergotoxine was merely to raise the arterial tension above a critical level at which adrenaline may produce a fall of pressure independently of ergotoxine, but Dale [1913] later showed that this view was erroneous because adrenaline could cause a fall of blood pressure after ergotoxine when the blood pressure was lower than it had been before ergotoxine when adrenaline had produced a rise of pressure. The action of M.T.E. contributes to the problem of reversal in so far as it shows that (vide Fig. 3) not only can reversal occur at a subnormal level of blood pressure, but also with a substance which produces no primary rise of pressure. That reversal of the motor effect of adrenaline is independent of any previous stimulation of smooth muscle by the reversing agent is also shown in the case of the rabbit's uterus.

With regard to the heart, it was originally shown by Dale [1906] that 'the cardio-accelerator effect of adrenaline is not abolished by such doses of ergot as suffice to reverse the effect on blood pressure', but that very large doses could abolish the accelerator myoneural junctions. Other observers have also found that the sympathetic terminations in the heart show a high degree of resistance to paralysis by ergotoxine. I have done many experiments on the perfused hearts of frogs and rabbits in which the hearts have been perfused with high concentrations of ergotoxine for long periods but have not found it possible to prevent the accelerator-augmentor effect of adrenaline in the concentration used. I have not

When the muscle had relaxed to about its normal tone (5 min. later) adrenaline (1 in 5,000,000) was again added (A_2), but this now failed to evoke any contraction.



Fig. 10. Isolated spleen of rabbit; showing motor effect of adrenaline 1 in 5,000,000 at A_1 , depressant action of M.T.E. 1 in 50,000 at M , and subsequent suppression of the motor effect of adrenaline at A_2 .

DISCUSSION

'*Sympathicolytic*' action. From the physiological point of view, the most interesting action of morphothebaine-dimethylether is that by which it can antagonize the motor effects of adrenaline on the smooth muscle of certain organs. Several substances are now known to exert this particular type of action to a greater or less degree, some of them, e.g. ergotoxine, producing an actual reversal of the motor action of adrenaline on certain tissues, others reducing or annulling the motor action of adrenaline without producing any reversal.

It would involve too wide a discussion to attempt to compare the actions of morphothebaine-dimethylether with those of all other substances which possess this type of action, but it may be of interest to note one or two differences between its actions and those of ergotoxine [Dale, 1906, 1913]. Unfortunately, the available supply of the new compound was only sufficient to permit the determination of its actions on a limited number of organs and species so that no complete comparison with ergotoxine is possible.

On such organs as have been investigated which normally give a motor response to adrenaline, M.T.E. in sufficient concentration readily reduces or suppresses that motor response. This effect is seen in blood pressure, heart, uterus, and spleen. In the intact animal, after intravenous injection of M.T.E., this effect is very transient and may last only a few minutes, in contrast to what happens with ergotoxine. This brevity

on blood pressure, pregnant uterus, or spleen, though ergotoxine produces adrenaline reversal in all these organs in this animal. On the other hand, adrenaline reversal has been obtained in the rabbit on blood pressure and on the uterus, whereas ergotoxine produces only slight, if any, reversal in these organs in this species.

SUMMARY

1. The actions of morphothebaine-dimethylether (M.T.E.) are described. This alkaloid is 3, 4, 6-trimethoxy-aporphine, closely related to bulbocapnine and corydine, less closely to apomorphine.

2. The L.D.₅₀ for mice by intraperitoneal injection is 0.11 g./kg., and for frogs, by injection into the dorsal lymph sac, 0.25 g./kg.

3. In mice M.T.E. produces in large doses clonic convulsions followed by central motor paralysis, with death from respiratory failure. The symptoms suggest a primary stimulation of the central nervous system above the spinal cord. In frogs no convulsions occur, and the symptoms suggest merely central motor paralysis. Small doses stimulate the respirations in mammals.

4. M.T.E. lowers the blood pressure in rabbits and cats, and depresses involuntary muscle of the heart, vessels, uterus, spleen and intestine.

5. M.T.E., in sufficient concentration, reduces or abolishes the motor effects of adrenaline on blood pressure, heart, uterus, and spleen. In the rabbit it may reverse the pressor effect of adrenaline on blood pressure and the motor effect on the uterus.

6. In this sympathicolytic action it resembles ergotoxine in many ways, but differs from the latter alkaloid mainly (a) in having no primary stimulant action on smooth muscle, (b) in paralysing more readily the peripheral cardio-accelerator mechanism in the heart, (c) in causing 'adrenaline reversal' on the blood pressure and uterus of the rabbit and not in the cat, and (d) in having a very transient action in the intact animal.

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been able, for example, to obtain with ergotoxine a result similar to that shown by M.T.E. in Fig. 5. Also it can be seen from Fig. 4 and Table 1 that adrenaline produced in the spinal cat only about half the percentage increase in cardiac rate after M.T.E. that it had produced before M.T.E., which suggests that M.T.E. cuts out the cardiac accelerator effect of adrenaline more effectively than ergotoxine would do in similar circumstances. The evidence is not conclusive but strongly suggests that M.T.E. has a relatively greater paralysing effect on the cardiac sympathetics than has ergotoxine.

In their actions on the uterus, there are also certain differences between the actions of ergotoxine and M.T.E., both in their primary actions and in their effects on adrenaline. Ergotoxine stimulates uterine muscle, whereas M.T.E. has a purely depressant action. Dale found that ergotoxine could readily produce reversal of the motor effect of adrenaline on the pregnant cat's uterus but slight, if any, reversal on the uterus of the rabbit. I have in a few experiments failed to obtain, after M.T.E., reversal of the motor effect of adrenaline on the pregnant cat's uterus. On the other hand, in certain rabbit's uteri a convincing reversal is obtainable with M.T.E., as in Fig. 7. It is generally believed that the sympathetic supply of the rabbit's uterus is purely, or almost purely motor [Dale, 1913]. The response of the isolated rabbit's uterus to adrenaline is a reaction which I have witnessed, either as a personal or class experiment, over 200 times and in only two cases have I come across a uterus which gave a purely inhibitory response to adrenaline, and in both those cases all segments of the uterus which were tested gave a pronounced inhibitory response. The infrequency of the incidence of this abnormal response in so large a number of unselected experiments makes it unlikely that an inhibitor response occurs normally at some critical period of the oestrous cycle. It must be regarded rather as an unexplained idiosyncrasy. This rare occurrence does, however, show that, according to the usual explanations, all rabbits' uteri are not devoid of inhibitor sympathetic fibres and, therefore, the reversal of the motor effect of adrenaline produced by M.T.E. in some rabbits' uteri is the less surprising.

Dale [1906] obtained a slight reversal of the motor effect of adrenaline following ergotoxine on the cat's spleen *in situ*. I have not obtained any reversal of the action of adrenaline following M.T.E. in the isolated spleen of the cat or rabbit, the action of M.T.E. in both cases being merely to obliterate the motor effect of adrenaline.

So far as my experiments have gone, therefore, they have failed to show any reversal of the motor actions of adrenaline in the cat, either

secretory granules. A thorough study of these problems by Mellanby [1925] led him to differentiate sharply between the functions of vagal and secretin stimulation, and to suggest that the metabolism of the enzymes of the pancreas was controlled by the vagi, while the chief, if not the only, effect of secretin was to cause the secretion of the water and inorganic constituents of the juice. According to this view, the granules discharged during secretin secretion are merely washed out by a dilute solution of sodium bicarbonate, the secretin exerting no direct effect upon them. This interpretation, although providing an attractive explanation of the dual mechanism, is in its turn difficult to reconcile with recent work involving the use of secretin as a clinical test for pancreatic function. Ågren & Lagerlöf (1936) and Diamond, Siegel, Gall & Karlen [1939] found evidence in man for a specific action of secretin upon the enzymes, shown most clearly by a rise in enzyme concentration at a time when the rate of flow of the secretion is diminishing after the initial rise in rate which immediately follows the secretin injection. Voegtlin, Greengard & Ivy [1934] also found that the effect of secretin in the dog and man was not only an increase in the rate of flow and of the output of enzymes, but in some cases an increased concentration of the latter. Finally, Langstroth, McRae & Komarov [1939], in a mathematical approach, have concluded that the mechanisms responsible for the secretion of protein and water in the pancreatic juice of the dog under secretin stimulation are largely independent, except in so far as both depend on secretin for their initiation. They did not investigate the enzyme content of the juice, but if it be assumed that this would largely parallel the protein content, their results are apparently not reconcilable with a passive washing-out of the enzyme material by the fluid. In view of these contradictory interpretations, it has seemed desirable to secure a more precise definition of the mechanism of the action of secretin, and it has been the object of the present investigation to provide this.

MATERIAL AND METHODS

Acute experiments were carried out on cats, which were anaesthetized with an intraperitoneal injection of nembutal (approximately 0.5 grain/kg.), prolonged anaesthesia being maintained, when necessary, by additional intravenous injections. Cannulae were inserted into the right femoral vein, the trachea and the pancreatic duct, while the pylorus and both carotid arteries were tied. In some experiments the bile duct was also cannulated, in order to remove any possible influence of the bile salts upon the course of the pancreatic secretion [Mellanby, 1928], although

THE INFLUENCE OF SECRETIN ON PANCREATIC SECRETION IN THE CAT

By E. J. W. BARRINGTON (*Rockefeller Foundation Fellow*)

*From the Department of Physiology, McGill University, Montreal, and
Department of Zoology, University College, Nottingham*

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DESPITE the extensive work which has been carried out on the properties of secretin, there still remains a fundamental uncertainty regarding its mode of action, arising from the existence in mammals of a dual mechanism controlling pancreatic secretion, comprising the parasympathetic secretory fibres running in the vagus, stimulation of which was shown by Pavlov [1897] to evoke a secretion of pancreatic juice, and the hormone secretin, discovered later by Bayliss & Starling [1902]. The latter concluded that the juice obtained by secretin injections was 'normal', with a full equipment of enzymes, and that the nervous mechanism was therefore superfluous, but this view became difficult to reconcile with certain later work which indicated that the output of enzymes was associated especially with vagal stimulation or with the parasympathomimetic action of pilocarpine. Bayliss & Starling [1904], together with de Zilwa [1904], themselves showed that the 'pilocarpine' juice contained a greater quantity of protein solids than did the 'secretin' juice. More recently Still & Barlow (1927) and Zucker, Newburger & Berg [1932] have confirmed this and have shown that the pilocarpine juice also has a higher enzyme content, while Crittenden & Ivy (1937) have shown that the dog's pancreas secretes continuously in the absence both of the secretin mechanism and of secretagogue absorption. Meanwhile Babkin, Rubashkin & Savitsch [1909] showed from cytological observations that an extensive extrusion of granules occurred during vagal stimulation, but that after copious secretion induced by hydrochloric acid acting on the duodenum the cells remained full of fine

which all the results are expressed. The methods are outlined briefly below:

Amylase. Following the procedure of Ågren & Lagerlöf [1936], a buffered starch solution was made up to contain

Dibasic sodium phosphate	2.37 g.
Monobasic potassium phosphate	2.27 g.
Sodium chloride	3.75 g.
Soluble starch	5 g.
Water	to 500 c.c.

Aliquot parts of the diluted juice were placed in 25 c.c. Erlenmeyer flasks, 25 c.c. of the starch solution added to each, and the mixtures incubated at 37° C. for 2½ hr. The flasks were then transferred to a refrigerator for 1 hr., and the reducing sugar in 1 c.c. of each mixture then immediately estimated by the method of Hagedorn & Jensen. In the tables and figures, the amylase concentration represents enzyme units per 1 c.c. of digest mixture; for convenience of tabulation, the total output of amylase in each portion of juice is expressed as one-tenth of the actual value.

Trypsin. A substrate solution was made up to contain 5% casein and 6% normal sodium hydroxide. To 25 c.c. of this were added 10 c.c. of veronal-sodium buffer at pH 8.4 [Weil, 1936], 2 c.c. of a 1 in 10 dilution of juice, and a few drops of toluene. The digest mixtures were incubated at 37° C. for 15 hr.; 10 c.c. of each were then added to 10 c.c. of distilled water in Erlenmeyer flasks, and the amino-acid content estimated by the formol-titration method, using 0.2 *N* sodium hydroxide. The trypsin concentration is expressed as units per 10 c.c. of digest mixture.

Lipase. A substrate solution was made up to contain 10% triacetin and 0.08% sodium glycocholate [Anrep, Lush & Palmer, 1925]. To 2 c.c. were added in a test tube 1 c.c. of Clark & Lubs' phosphate buffer at pH 8, 1 c.c. of a 1 in 10 dilution of juice, 4 drops of phenol red and 1 drop of toluene. The digest mixtures were incubated at 37° C. for 24 hr., and then titrated back to pH 8 with 0.05 *N* sodium hydroxide. The lipase concentration is expressed as units per total volume of digest mixture.

Reliance has been placed chiefly on the estimation of amylase, as the selected method gave much greater sensitivity than could be obtained for the other enzymes. Where the latter were also estimated, the general shapes of the curves for the several enzymes were in close agreement, in accordance with the generally accepted principle of the parallel secretion of the pancreatic enzymes. That this parallel secretion occurs during secretin stimulation is shown in Fig. 1; such slight variations as were

no significant differences were noted when this procedure was adopted. Unless otherwise stated, both vagi were cut.

Secretin solutions, adjusted in concentration to the weight of the animal, were injected into the femoral vein in volumes of 1 or 2 c.c. at intervals of 5, 10 or 15 min. according to the nature of the experiment. It is well known that much of the earlier work on the action of secretin suffered from the use of relatively crude preparations containing histamine-like substances and other vaso-depressors which in themselves affect the pancreatic secretion, and it is always open to argument that the enzyme content of the juice in such experiments might result from the action of such substances rather than of secretin itself. Care was therefore taken to ensure, by blood-pressure tests, that the secretin used in the present work had no significant vaso-depressor action, while the following different preparations were used in order to give the results as wide a significance as possible:

Secretin A¹ and A². Two preparations of the secretin concentrate 'SI' of Ivy, Kloster, Drewry & Lueth [1930]; A² (in solutions of 1.0 mg./c.c./3 kg.) was free of vaso-depressors, while A¹ included not more than an unimportant trace. These were used for the majority of the experiments.

Secretin B. The commercial 'Pancreotest' preparation supplied by Messrs Astra, of Sweden. This is a highly purified substance used for clinical tests, and is guaranteed free from antigens, cholecystokinins and vaso-depressors.

Secretin C. A highly purified material prepared by Dr S. A. Komarov from the intestine of the dog. After precipitation by sodium chloride and by trichloroacetic acid, subsequent purification followed the suggestions of the Swedish workers [Hammarsten, Ågren & Wilander, 1933], and involved successive precipitation by mercuric sulphate, picrolonic acid and picric acid, the final precipitate being converted into the chloride. In some tests this material appeared to be free from vaso-depressors, but others suggested the presence of a small trace which would not be significant for the present work.

Secretin D. A crystalline material prepared by Dr Komarov from the 'SI' concentrate according to the procedure of Greengard & Ivy [1938]. This was a very potent preparation, free from vaso-depressors (0.1 mg./c.c./3 kg.) but containing more than one type of crystal.

The pancreatic juice was collected, usually in 15 or 30 min. portions, in graduated tubes on ice, and was stored in a refrigerator overnight. All digest mixtures were made up within 24 hr. of the collection of the juice. Standard methods were used for the estimation of the enzymes in the samples, suitably modified so as to provide reasonable sensitivity over the considerable range of concentrations found in the experiments. Standard curves were constructed for each enzyme using a known series of dilutions of a portion of juice, and from these curves all titration readings were transformed into arbitrary enzyme units in

ACTION OF SECRETIN ON PANCREATIC SECRETION 85

TABLE 1. Cat 11, weight 3.7 kg.; 2 c.c. secretin A¹ (0.8 mg./c.c.) each 15 min.:
juice collected in 30 min. portions (1-12)

	1	2	3	4	5	6
Volume (c.c.)	4.0	3.25	3.25	3.4	3.6	3.9
Amylase concentration	136.2	63.5	60.0	53.5	52.0	43.0
Amylase output	27240	10320	9550	9080	9560	8385
	7	8	9	10	11	12
Volume (c.c.)	3.95	3.80	3.85	3.9	4.0	4.0
Amylase concentration	38.0	35.5	27.5	23.5	21.5	19.0
Amylase output	7055	6745	5295	4585	4300	3800

of enzymes in the juice. The progressive fall in enzyme output is characteristic of such experiments, and agrees with the protein-nitrogen readings for the pancreatic juice of the dog obtained by Komarov, Langstroth & McRae [1939] under comparable experimental conditions. Similar results were obtained in other experiments, including ones in which secretin C was administered for 4½ hr. and D for 5½ hr.

At any stage during such an experiment faradic stimulation of the vagus will cause an increase in both the concentration and total output of enzymes. This is shown in Table 2, where portion 9 was the result of

TABLE 2. Cat 21, weight 3 kg.; 2 c.c. secretin A² (1 mg./c.c.) each 15 min.; juice collected in 30 min. portions (1-9). After taking portion 8, each vagus stimulated for 1 min. (coil 10), repeated after 15 min.

	1	2	3	4	5	6	7	8	9
Volume (c.c.)	2.1	1.9	1.8	2.3	2.2	2.4	2.5	2.2	2.7
Amylase concentration	15.0	11.5	9.5	8.5	8.5	8.0	8.5	7.5	16.5
Amylase output	1575	1093	855	978	935	960	1063	825	2228
Lipase concentration*	69.0	63.0	51.5	51.5	51.5	55.0	55.0	48.5	110.0
Lipase output	1449	1197	927	1185	1133	1320	1375	1067	2970

combined vagal and secretin stimulation and shows a markedly increased activity over the previous portions which were the result of secretin stimulation alone. This parasympathetic effect, already referred to, has been taken as evidence that the fall in enzyme output during secretin stimulation is not due to a true exhaustion of the gland. Anrep *et al.* [1925] believed that the changes in the concentration of the enzymes in similar acute experiments on the dog depended upon the general condition of the animal and of the blood supply to the gland, low enzyme concentration being associated with low blood pressures, but they used a crude secretin containing vaso-depressors, and their conclusion is not applicable to the present work. Mellanby [1925], using a much purer preparation, regarded the effect as supporting his view that the enzyme output was determined by the parasympathetic stimulation and not by the secretin. However, another explanation would be that the output

noted in the present work need indicate nothing more than differences in sensitivity of the methods, or the absence of optimum activating conditions in the digest mixtures, although there is some evidence that under pathological conditions this parallelism may break down [Diamond *et al.* 1939].

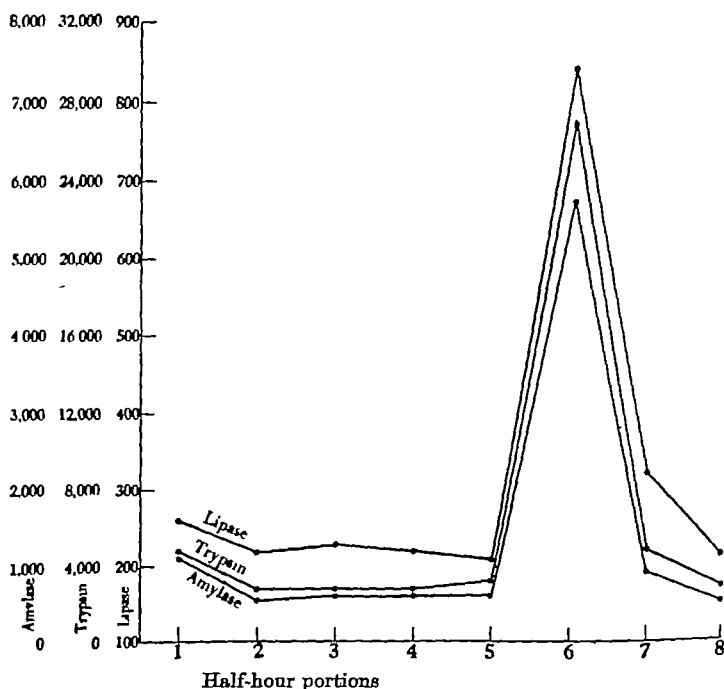


Fig. 1. Cat 23, weight 3.1 kg. Parallel fluctuations in output of amylase, trypsin and lipase during secretin administration. 1 c.c. of secretin A² injected each 10 min., except for portion 6, for which the rate was quadrupled. Juice collected in 30 min. portions. Ordinates: Enzyme output in arbitrary units.

In order to check the condition of the pancreas at the end of some of the experiments, small pieces of the organ were fixed overnight in Helly's fluid, embedded in wax and sectioned in the usual way, and stained in iron haematoxylin.

EXPERIMENTAL

The volume and enzyme content of the juice obtained by repeated administration of secretin (A¹) over a period of 6 hr. is shown in Table 1. This experiment, begun after an hour's restitution which followed upon several secretin injections, clearly demonstrates that after 6 hr. of secretion under secretin stimulation there is still a readily measurable quantity

in Table 1. It is probably significant that this experiment was begun with the pancreas already considerably exhausted after a series of secretin injections not recorded in the table. Clearly, whatever may be the correct explanation of this constancy, continued fall in output is not an inevitable accompaniment of secretin administration, and the possibility of secretin directly stimulating the discharge of enzymes cannot, therefore, be excluded.

Mellanby [1925] showed that section of the vagi during repeated administration of secretin caused a considerably diminished output of enzymes, and held this as further support for the view that the output was determined by vagal impulses. An experiment of this type is shown in Table 4; the vagi were cut after the collection of portion 5, and the

TABLE 4. Cat 10, weight 3 kg.; 2 c.c. secretin A¹ (0.6 mg./c.c.) each 15 min.; juice collected in 30 min. portions (1-10). Vagi cut after collecting portion 5

	1	2	3	4	5	6
Volume (c.c.)	1.9	2.1	2.25	2.3	2.05	1.55
Amylase concentration	71.0	48.0	33.5	29.0	27.5	24.0
Amylase output	6745	5004	3770	3330	2820	1860
Trypsin concentration	—	145.0	105.0	100.0	100.0	97.5
Trypsin output	—	5633	4168	4255	3793	2795
	7	8	9	10	11	12
Volume (c.c.)	1.45	1.4	1.5	1.75	1.80	1.75
Amylase concentration	19.5	19.5	17.5	16.5	13.0	12.0
Amylase output	1415	1365	1315	1445	1170	1050
Trypsin concentration	62.5	77.5	77.5	72.5	67.5	50.0
Trypsin output	1676	2152	2152	2348	2248	1619

results confirm the occurrence of some reduction in output. The significance of this is not, however, quite clear, since the rate of flow also diminished and later increased, possibly as a result of the mechanical irritation stimulating the vagal endings in the smooth muscle fibres of the pancreatic ductules [Anrep, 1916]. In any case, the essential fact is that the discharge of enzymes is still continuing, at only a slowly diminishing rate, 3½ hr. after the section of the vagi, and this, in conjunction with the numerous experiments in which the nerves were cut from the beginning, indicates that secretin can effect discharge of enzymes in the absence of vagal impulses, even although the presence of the latter may augment that discharge.

Finally, in order to eliminate the possibility that the enzyme content of the juice during prolonged secretin stimulation with the vagi cut might be due to stimulation of the vagal endings by parasympathomimetic substances in the secretin preparation rather than to the action of the secretin itself, some experiments were carried out upon atropinized

depends upon both the intensity of the secretin or other stimulus, and also upon the amount of zymogen present in the gland cells. If this were so, under constant secretin stimulation the enzyme output would fall as a result of the progressive reduction of the zymogen content of the gland, a line of argument which has received interesting mathematical treatment at the hands of Langstroth *et al.* [1939], although, as they themselves clearly recognize, certain of their assumptions may be over-simplified.

This last hypothesis suggests two possible consequences. First, variations in the intensity of the secretin administration should produce variations in output, and that this does in fact occur is shown in Table 3.

TABLE 3. Cat 7, weight 3 kg.; 2 c.c. secretin A¹ (0.6 mg./c.c.) each 5 min. (portions 1-4), then 1 c.c. each 10 min. (portions 5-8), then 2 c.c. each 5 min. (portions 9-12); juice collected in 30 min. portions. After taking portion 8, faradic stimulation (secondary coil at 10 cm.) applied for 1 min. to each vagus and repeated after 15 min. interval. Total period of stimulation, 4 min.

	1	2	3	4	5	6
Volume (c.c.)	2.85	2.65	2.80	3.15	2.75	2.35
Amylase concentration	30.0	12.0	10.0	8.0	4.5	4.5
Amylase output	4275	1590	1400	1260	620	530
	7	8	9	10	11	12
Volume (c.c.)	2.15	1.8	3.05	3.45	3.75	4.0
Amylase concentration	5.5	6.0	6.0	6.0	5.0	4.5
Amylase output	565	540	915	1035	940	900

Here, in the first phase of the experiment (portions 1-4), there was the usual fall in output: in the second phase (portions 5-8) the rate of secretin injection was reduced to one-quarter of the previous rate, and this was accompanied by a fall in concentration and output and a more gradual but equally clear fall in volume; in the third phase (portions 9-12) the secretin injections were restored to their original rate, and there was an immediate increase in output and volume.

A second possible consequence is that after prolonged administration of secretin, with corresponding loss of zymogen from the gland, the falling output might increasingly approximate to the rate of synthesis of fresh material, and would finally become constant, output and synthesis balancing each other. In practice, the animals are found to vary so much in their sensitivity to secretin that there is no certainty of attaining this stage without the use of an extravagant amount of secretin, but in certain experiments the output has actually been found to maintain relative constancy over a number of samples. Table 2 shows such a condition extending over 3½ hr. (portions 3-8), during which the enzyme output fluctuated around a constant level rather than showing a steady fall as

Such observations, however, cannot be conclusive, and it has seemed more profitable to devise some means of dissociating the rate of flow from the secretin stimulus by varying the former while maintaining the latter constant. For this purpose, advantage has been taken of the secretagogue action of sodium nitrite, the effect of which in augmenting pancreatic secretion appears to be a result of vasodilatation [Barlow, 1927].

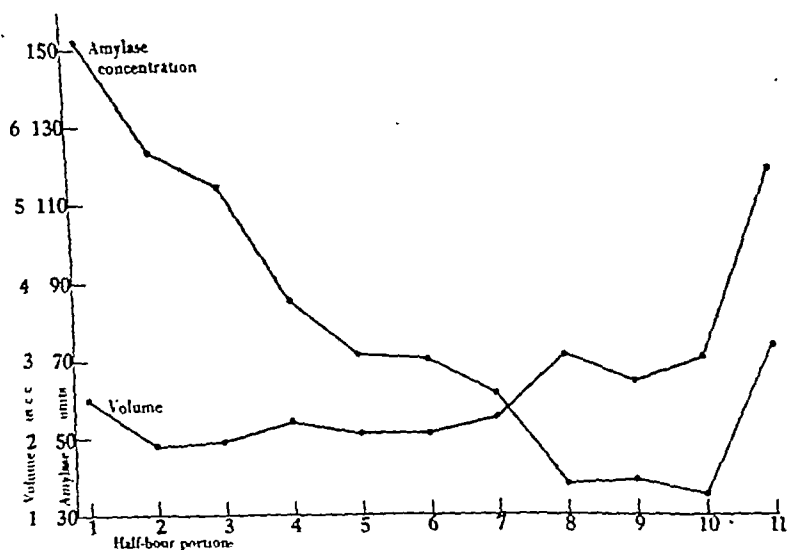


Fig. 2. Cat 18, weight 3 kg. Effect on volume of secretion and on amylase concentration of injecting secretin A¹ and sodium nitrite. Juice collected in 30 min. portions. Here and in Fig. 3 the total amylase output in each portion of juice is: volume \times concentration \times 400. For further explanation see text.

One experiment involving the use of this salt is shown in Fig. 2. Portions 1-7 (inclusive) were taken during a constant rate of injection of secretin, and show the usual decline in enzyme concentration and output. During the period represented by portions 8-10, sodium nitrite (3 mg./15 min.) was added to the secretin. The result was an increased rate of flow (volume) but a further diminution of total enzyme output and therefore a fall in concentration. Portion 9 is particularly striking, for here, by accident, slightly less secretin than normal was injected, and this was marked by a slight fall in volume and output. Finally, portion 11 was the result of a maintenance of the nitrite injections at the same rate, but a quadrupling of the secretin injections; here there was a sharp rise in volume, concentration and output. In this experiment, then, which has been confirmed, the rate of flow was increased in two different ways, first

animals. The atropinization was checked by faradic stimulation either of the submaxillary gland through the chorda tympani, the duct of the gland being cannulated, or of the pancreas through the cut vagi, a portion of juice being collected in the latter case and tested for the absence of any increase in enzyme output. The result of one of these experiments is seen in Fig. 3, which shows that enzymes continue to be secreted for $5\frac{1}{2}$ hr. even during paralysis of the parasympathetic nerve endings.

It may be concluded from the experiments recorded above that pancreatic juice secreted under the stimulus of secretin contains enzymes which are discharged independently of any vagal stimulus, but this conclusion still sheds no light upon the fundamental question as to whether that discharge is a result of direct action of secretin upon the cell inclusions or merely of the washing-out of the granules by the fluid passing through the cell. It is therefore necessary next to inquire whether any relationship can be traced between the rate of flow and the output of enzymes, for if the latter are merely washed out by the bicarbonate solution then an increased rate of flow might be expected to result in an increased output of enzymes per unit time. Indeed, it has already been seen (Table 3) that a diminished rate of flow may be associated with a fall in output.

The most convenient way of varying the rate of flow of the fluid is by varying the rate of injection of secretin, as shown in Table 3. During the second phase of this experiment, when the secretin stimulation was reduced to one-quarter of the previous rate, the volume, concentration and output were all lowered, but it is noticeable that while the output fell abruptly, the volume (i.e. rate of flow) fell much more gradually and there is thus little indication of any direct relationship between them. With a resumption of the original rate of injection (portions 9-12) there was a sharp increase in volume and total output, but again with no clear relationship between them, for the latter fell slightly while the former continued to increase (portions 10-12). Other experiments show a further point, not strikingly shown in Table 3, and that is that an increase in the rate of injection may produce an increase in concentration of the enzymes as well as of volume and total output. This is well seen in Figs. 2 and 3, where the rate of secretin injection was quadrupled for portions 11 and 6 respectively. Such an effect of secretin on concentration is strongly suggestive of a direct action upon the cell inclusions rather than of an indirect effect of washing-out, and Diamond *et al.* (1939), who found secretin to affect the concentration of enzymes in human pancreatic juice, have argued in the same way.

volume, concentration and output. Calculation shows that the total output in portion 6 is 4.4 times that in portion 5, while that in portion 12 is 3.9 times that in portion 11; these increases are of the same order, and there is thus no indication of any inhibitory effect induced by the nitrite. As may be seen from Fig. 3, the increase in concentration in portion 12 as compared with 11 is considerably greater than that in 6 compared with 5. Finally, it may be noted that the increased volume seen in portion 3, and due apparently to some effect of the atropine [Mellanby, 1925], is not accompanied by any increase in concentration or output.

It may be concluded from these and similar experiments that an increase in the rate of secretion of pancreatic juice is not necessarily accompanied by any increase in the output of enzymes, but that the latter is determined, independently of the rate of flow, by the direct action of secretin upon the cell inclusions.

DISCUSSION

There is little to add by way of discussion to the above account. It has been shown that secretin, as much as the vagus nerves, has a stimulatory effect upon the inclusions of the pancreatic cells and therefore plays a direct part in the control of enzyme output. This is a conclusion of some interest for the comparative physiologist, for it has been suggested [Babkin, 1931] that in the skate the pancreatic secretion may not be under nervous control. In lampreys the zymogen cells corresponding to those of the pancreas of higher vertebrates appear still to be located in the gut epithelium [Barrington, 1936], and it is thus conceivable that the secretin mechanism may have evolved first as a local hormone [Collip, 1938], the nervous mechanism being added later. This, however, is quite speculative, and much further work would be needed to confirm any such suggestion.

It seems impossible to decide what the relative importance of the two stimuli may be in the normal feeding of the mammal, although there is no reason to suppose that it is mirrored by their apparent relative importance under certain experimental conditions, for while faradic vagal stimulation and pilocarpine injections are known to cause extensive discharge of zymogen granules, they may well represent a more vigorous stimulation than would be physiologically normal, and indeed it is known that after strong pilocarpine stimulation the pancreatic cells of the mouse require at least 3-4 hr. restitution before becoming capable of fresh extrusion [Hirsch, 1932]. The major difficulty in estimating the importance of secretin under physiological conditions lies in a lack of knowledge

by the injection of nitrite, the secretin stimulus remaining constant, and secondly by the increase of secretin, the nitrite remaining constant. The fact that only the second increase caused a rise in concentration and output of enzymes strongly implies that these are determined by the direct action of secretin and not by the rate of flow of fluid through the cell.

One objection to the above experiment suggests itself, and that is that the nitrite might exert some undefined inhibitory effect on the discharge of zymogen, and thus prevent the rise in output which would otherwise result from the increased rate of flow of fluid in portions 8-10. To meet

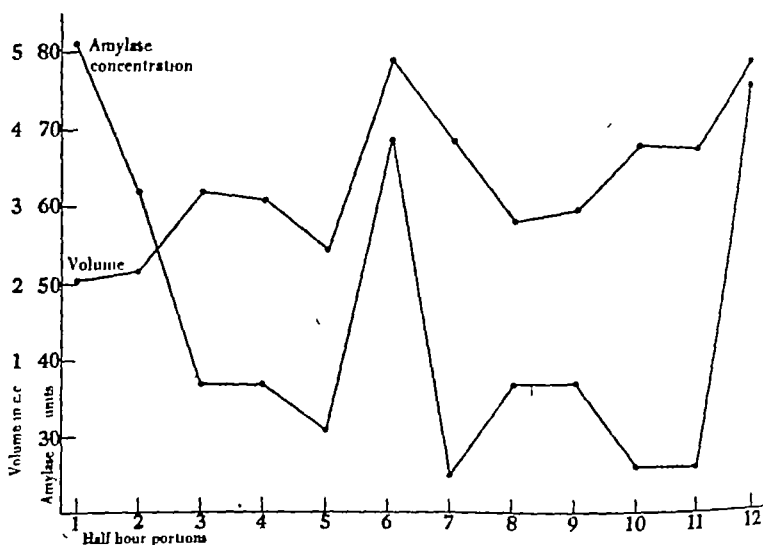


Fig. 3. Cat 19, weight 3.1 kg. Effect on volume of secretion and on amylase concentration of injecting secretin A² and sodium nitrite into an atropinized animal. Juice collected in 30 min. portions. 2 mg. atropine injected after collecting portion 1, 1 mg. after portion 5, 1 mg. after portion 11. For further explanation see text.

this, the experiment shown in Fig. 3 was devised, the animal being also atropinized to remove any possible vagal influence. Portions 1-5 (inclusive) were collected under constant secretin stimulation (1 c.c. each 10 min.). For portion 6 the secretin stimulus was quadrupled, and this resulted in a sharp rise in volume, concentration and output. Portions 7-9 were collected under the original secretin stimulus, and show a return to the original values. For portions 10 and 11 sodium nitrite (3 mg. each 10 min.) was added to the secretin, and this resulted in a rise in volume but not in concentration or output. For portion 12 the nitrite was maintained and the secretin quadrupled, and there was the expected rise in

for providing me with a sample of secretin, and for kindly preparing some crystalline material from the 'SI' secretin concentrate, and to Mr M. J. Schiffrin for assistance in the preparation of that concentrate.

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as to its effect upon the cell inclusions. It has been said that after prolonged secretin administration the cells of the pancreas are still in a 'resting' condition [Mellanby, 1925], but Netik [1937] has presented some evidence for a decided reduction of zymogen granules in the dog after such treatment. An examination of sections of material from some of the cats used in the present work shows that although an abundance of granules is still present, there is sufficient variation in the appearance of individual cells to accord with the assumption of a discharge of zymogen from them. Some variation has been noted in the zymogen content of the pancreatic cells of the mouse, apparently resulting from the spontaneous secretory activity of the 'resting' gland [Hirsch, 1932]. It is not desired to press this point here, or to emphasize the cytological picture, since there is an obvious need for a statistical study of the effects of secretin upon the cell in a more convenient animal than the cat, and such work is now in progress.

At present nothing can be suggested as to the history of the cells during their activity. It is essential first to know, for example, whether the effect of secretin is to cause a few cells to discharge completely their granules, or whether a large number of cells discharge a small proportion of their contents, and other questions readily suggest themselves. Until they have been answered, it must be emphasized that the suggestion put forward above, as to the setting up of a balance between synthesis and output, is essentially speculative, and is intended merely to aid discussion of the experimental results.

SUMMARY

In atropinized cats, or in cats with both vagi cut, enzymes continue to be discharged in the pancreatic juice after at least 6 hr. continuous secretion under secretin stimulation. A continued fall in enzyme output does not necessarily occur under these conditions.

If the rate of flow of the pancreatic juice is increased either by administration of sodium nitrite or by increasing the rate of secretin injection, only the latter results in an increased concentration and output of enzymes.

It is concluded that secretin directly stimulates the discharge of zymogen, and that the enzyme content of 'secretin' juice does not result merely from the passive washing-out of the zymogen by the fluid passing through the pancreatic cells.

The work was carried out at Montreal during the tenure of a Rockefeller Foundation Fellowship, and I am much indebted to Prof. B. P. Babkin for the hospitality of his laboratory and for his guidance and criticism. I am indebted also to Dr S. A. Komarov

for providing me with a sample of secretin, and for kindly preparing some crystalline material from the 'SI' secretin concentrate, and to Mr M. J. Schiffrin for assistance in the preparation of that concentrate.

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THE HAEMOPOIETIC ACTIVITY OF
MAMMALIAN LIVERSBY F. X. AYLWARD, W. S. M. GRIEVE,
B. R. S. MAINWARING AND J. F. WILKINSON*From the Department of Clinical Investigations and Research,
University and Royal Infirmary, Manchester**(Received 10 February 1941)*

It is now generally accepted that in the formation of red blood corpuscles chemical agents play an important part. One of these is the so-called anti-pernicious anaemia principle of the liver. This has not yet been separated in a chemically pure state, but evidence has been presented that the substance itself or a precursor of it is produced by the interaction of a gastric 'intrinsic factor' (haemopoietin) present in gastric juice and an 'extrinsic factor' present in certain foodstuffs [Castle, 1929*a, b*; Castle & Townsend, 1929; Castle, Townsend & Heath, 1930; Wilkinson & Klein, 1932; Klein & Wilkinson, 1933, 1934; Jones, Grieve & Wilkinson, 1938].

Since no chemical or biological test for the liver principle has been discovered, the only method for its assay in extracts of liver is that of clinical trial on suitable cases of pernicious anaemia.

By the use of this method the presence of the principle in normal human liver and its absence in cases of pernicious anaemia has already been demonstrated, as well as its occurrence in the livers of patients with pernicious anaemia who have responded to treatment but died of other diseases [Wilkinson & Klein, 1934; Wilkinson, Klein & Ashford, 1937]. Active liver extracts for therapeutic use have also been prepared commercially from calf, ox, sheep, pig and horse. In addition, the principle has been shown to be present in deer [Minot & Murphy, 1928] and dog livers [Strauss & Castle, 1933-4], whilst Connery [1930], Hanssen [1931], Davidson [1932] and Wilkinson [1933] have found it in considerable amount in fish livers.

The widespread occurrence of this active principle or principles is of great interest, since pernicious anaemia is not known to affect any animal other than man. Furthermore, the gastric factors involved in erythropoiesis apparently differ to some extent in carnivorous, omnivorous and herbivorous animals [Wilkinson, 1936]. It was therefore with the object of extending our knowledge of the occurrence of the liver principle that the present study was undertaken. Livers from a series of mammals (some domestic or indigenous to this country, others wild animals from different parts of the world) have been examined histologically and extracts have been prepared from them. These extracts have been tested on patients suffering from pernicious anaemia.

METHODS

Preparation of liver extracts

The livers were removed immediately after death, frozen in a refrigerator and extracted according to our standard technique. The liver was weighed, minced and then extracted with acidified 80% alcohol (1.3 l. 80% alcohol and 1 c.c. 50% sulphuric acid/kg. moist liver). After frequent stirring over a period of 24 hr. the mixture was filtered, the filtrate being placed aside and the residue re-extracted with 40% alcohol (2.5 l./kg. original moist liver) with frequent stirring as before.

The combined filtrates were evaporated under reduced pressure in a Reich apparatus at a temperature below 40° C. to a small volume (approximately one-fortieth of the original volume). An equal volume of absolute alcohol was added, and the resulting precipitate after being allowed to settle was removed by filtration. The filtrate was evaporated under reduced pressure to a syrup which was poured slowly with constant stirring into 10 vol. absolute alcohol and the resulting precipitate dehydrated with successive quantities of alcohol, washed with ether, dried in a vacuum desiccator and weighed.

The very hygroscopic powder was preserved in vacuo in a desiccator until required for the clinical tests, when a weighed amount was dissolved in the minimal amount of distilled water and sterilized by filtration through a Seitz filter into sterile ampoules.

This method furnished preparations with the minimum of manipulation, thus avoiding much loss of activity, a very important point when dealing with the livers of small animals.

A summary of the extracts obtained from the livers of twenty different animal species is shown in Table 1. For comparative purposes the data obtained from normal human livers [Wilkinson & Klein, 1934] is included.

TABLE 1. Mammalian liver extracts

Extract no.	Animal	Weight of liver g.	Weight of extract g.	Extract g./100 g. liver
	Herbivorous			
L.I. 90	Ox	2,000	37.5	1.9
L.I. 10	Ox	1,000	29	2.9
R. 79	Sheep	—	—	—
L.I. 112	Horse	2,000	12	0.6
L.I. 67	Indian elephant	2,450	31.7	1.3
L.I. 76	African rhinoceros	3,010	36.6	1.2
L.I. 102	African rhinoceros	18,200	253	1.4
L.I. 74	Giraffe (Baringo variety)	1,000	8.8	0.9
L.I. 101	Nylghaie antelope (blue bull)	2,400	7.0	0.3
L.I. 100	Gibbon	205	2.0	0.9
L.I. 107	Gibbon	—	—	—
L.I. 109	Green monkey	140	1.5	1.1
L.I. 94	Red-faced Japanese monkey	270	7.5	2.8
L.I. 96	Chimpanzee	630	18.0	2.9
L.I. 64	Orang-utan	1,640	14.5	0.9
	Omnivorous and carnivorous			
L.I. 2	Man	1,500	34	2.3
L.I. 6	Man	950	16.5	1.7
L.I. 7	Man	1,200	10	0.8
P. 1	Pig	—	—	—
P. 2	Pig	—	—	—
L.I. 110	Lion	1,000	5	0.5
L.I. 86	Lion	2,070	38	1.8
L.I. 105	Tiger	—	—	—
L.I. 106	Leopard	720	8.5	1.2
L.I. 104	Ocelot	200	2.3	1.1
L.I. 92	Californian sea-lion	1,770	5.6	0.3
L.M. 39	Californian sea-lion	1,700	17	1.0
	Whale	—	—	—
	Sus			
	Sus	—	—	—
	Sus	—	—	—
	Felis leo	—	—	—
	Felis tigris	—	—	—
	Felis pardus	—	—	—
	Otaria californiana	—	—	—
	Otaria californiana	—	—	—

Since this work has been carried out over a period of several years, as the material has become available, the method of preparation of the extracts has been repeatedly checked by us by many control extractions of fresh calf or bovine livers and re-testing them for anti-pernicious anaemia potency. These control results are omitted from the tables except for a few illustrative ones.

We have also examined from time to time many commercial preparations made from cattle, pigs, sheep, horse, whale, etc.

Tests for activity

The difficulties in assaying accurately the liver principle have been discussed by many workers, but in spite of the efforts which have been made to obtain a reliable chemical or animal test the clinical test as used in the present work remains the only test of any value [Wilkinson, 1932, 1933].

The potency of the extracts was determined by the haematological and clinical effects following their intramuscular injection, after preliminary control periods, into well-authenticated and specially controlled patients with relapsing pernicious anaemia. Haematologically active extracts produce first a sharp rise or peak in the number of reticulocytes in the circulating blood, within 6-10 days, and this is followed by an increase in the number of red blood cells and in the percentage of haemoglobin, with a simultaneous improvement in the clinical condition. The patients used for this purpose were all carefully chosen according to the rigid criteria laid down by one of us [Wilkinson, 1932]; estimations of the reticulocytes were made daily, and of the red cells, white cells and haemoglobin, once or twice weekly as indicated by the condition of the patient.

Even with this test difficulties arise in expressing the activities of the extracts in a strictly quantitative manner. It is desirable that each extract should be tested on as many patients as possible and that the amount of extract given should be varied, so that the minimal amount necessary to produce a maximal reticulocyte response can be ascertained. Such a procedure is usually impossible because of the large number of extracts to be tested, the relatively small number of suitable patients available and, in the present work, because of the very limited amount of available material from some of the smaller animals.

Stained paraffin sections of all the livers were examined histologically.

RESULTS AND DISCUSSION

The yields of the extracts varied between 0.3 and 2.9 g./100 g. fresh liver tissue (Table 1), and even within the same species the yields were by no means constant as we had observed in our previous work on human and bovine livers [Wilkinson & Klein, 1934]. Because of these variations

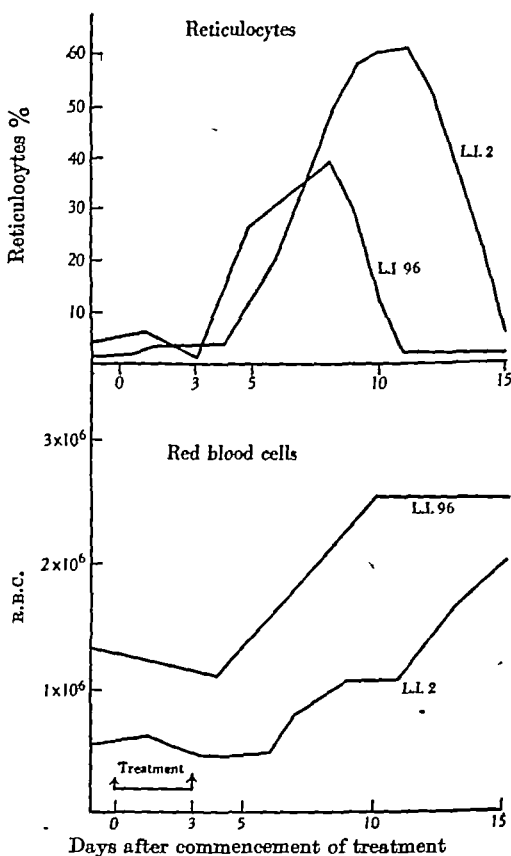


Fig. 1. Response to injections of human (L.I. 2) and chimpanzee (L.I. 96) liver extracts.

the amounts of the extracts administered to the test patients (Tables 2 and 3) have been expressed in terms of moist liver weight, since these figures are of greater comparative value. We were unable to use equal or equivalent amounts of extracts in all cases on account of the limited quantities of some of the livers at our disposal. Some typical examples are shown in Fig. 1, in which liver extracts L.I. 2 (man) and L.I. 96 (chimpanzee) were given to test cases 398 and 936 respectively.

The results of the clinical tests are summarized in Tables 2 and 3, from which it will be seen that an anti-pernicious anaemia principle is present in the livers of a wide variety of herbivorous and omnivorous and to a less extent in the carnivorous mammals examined.

When comparing the activity of livers from various sources it is necessary to consider among other things the dosage of liver extract, the degree of reticulocytosis in relation to the original red cell count, and the rate of increase in the red cell count. In general the smaller the initial red cell count the larger will be the response from a standard amount of liver extract; this inverse relationship is well illustrated in Fig. 1. Doses of liver greater than a certain optimal value do not give greater increases in the reticulocyte or red cell count.

With the herbivorous mammals (Table 2) it will be noted that all gave good haematological responses, initiating active remissions in test patients with pernicious anaemia.

Of the omnivorous mammals (Table 3), normal man [see Wilkinson & Klein, 1934] and the pig possessed livers containing large quantities of the anti-pernicious anaemia principle of about the same order as the herbivora.

In the group of carnivorous animals active clinical responses were obtained with liver extracts from lion, tiger, leopard and ocelot.

The whale-liver extract possessed good anti-pernicious anaemia activity, but on the other hand, the livers from the two sea-lions failed to yield haemopoietically active extracts (L.I. 92, 104), although very slight reticulocyte increases without red cell responses were noted in one of the three test cases.

Age, diet and cause of death of the animals will play some part in determining the amount of anti-pernicious anaemia factor in the livers. We would, therefore, expect differences in herbivorous, carnivorous and omnivorous animals, but the data yield little evidence on this point. This is in contrast to the differences that have been observed in stomach preparations from herbivorous and other animals. The activity of human gastric juice and gastric mucosa has been demonstrated frequently [Castle, 1929*a*; Wilkinson *et al.* 1938]. Commerical stomach preparations are usually obtained from the pig [Wilkinson, 1930], but active material has also been obtained from other animals such as the silver fox [Wilkinson, 1933]. The herbivorous animals, on the other hand, appear to give stomach preparations of little or no activity [Wilkinson, 1933], as we have also shown using the desiccated stomachs of the sheep, horse, rhinoceros, elephant, etc.; Reimann, Steiner & Grunfield [1936-7] also failed to find activity in desiccated horse stomach.

TABLE 2. The effects of extracts prepared from livers of herbivorous mammals

Animal	Liver extract no.	Extract used in terms of fresh liver g.	Case no.	Period of treatment days	Reticuloocytes		Initial counts		Later counts		Day of treatment
					Max. %	Day of response	R.B.C.'s $\times 10^3$	Hb %	R.B.C.'s $\times 10^6$	Hb %	
Ox	90	280	902	2	28.6	7	1.19	32	2.03	50	10
	10	1000	396	5	55.0	0	0.70	19	1.47	40	14
	10	240	771	3	21.0	6	1.19	33	2.62	60	21
	10	160	774	2	30.0	7	1.06	34	2.40	60	21
Sheep	79	160	777	2	32.2	8	1.50	48	2.74	66	18
	70	200	705	4	25.4	10	1.80	39	2.29	54	19
Horse	112	500	1065	2	23.2	6	1.33	34	2.04	68	10
	112	500	383	2	19.8	6	1.55	50	2.90	68	17
	112	500	1072	2	16.5	7	1.90	52	2.72	67	14
	67	776	1065	5	50.0	10	1.45	36	2.81	60	20
Elephant	67	386	809	3	45.0	9	1.35	30	2.62	50	15
	67	310	778	1	23.0	7	0.81	20	1.12	23	7
	76	372	843	2	39.7	8	1.41	34	2.30	53	22
	76	826	296	3	28.4	6	1.17	31	2.45	54	15
Rhinoceros	102	900	1030	3	17.4	6	1.06	50	3.12	79	15
	102	900	1021	3	24.2	10	1.03	29	2.57	61	21
	74	455	884	2	36.4	7	0.91	20	2.06	44	13
	101	2400	1024	2	28.2	7	1.20	36	2.09	52	19
Gibbon	100 + 107	95	1074	2	27.6	9	1.49	40	2.81	66	14
Green monkey	109	140	1080	3	28.5	9	2.31	67	2.06	74	10
Red-faced monkey	94	268	911	2	29.6	7	1.59	42	2.17	57	14
Chimpanzee	96	400	936	3	38.4	9	1.10	32	2.51	56	11
Orang-utan	64	988	508	11	28.0	8	0.75	18	2.50	47	24

TABLE 3. The effects of extracts prepared from livers of omnivorous and carnivorous mammals

Animal	Liver extract no.	Extract used in terms of fresh liver g.	Test case no.	Period of treatment days	Reticulocytes		Initial counts		Later counts		Day of treatment
					Max. %	Day of response	R.B.C.'s $\times 10^4$	Hfb %	R.B.C.'s $\times 10^4$	Hfb %	
Man	2 + 6	1235	384	7	42	11	1.39	35	2.00	53	15
	7	1200	392	4	30	7	1.12	31	2.46	54	15
	2	1015	398	4	62	10	0.43	13	2.00	30	15
Pig	P. 1	160	237	4	30	10	1.37	42	2.48	64	15
	P. 2	160	513	4	25.1	9	1.04	49	2.34	68	18
Lion	110	500	1010	2	—	—	1.83	52	3.27	85	14
	110	500	1072	2	20.4	8	1.28	34	1.03	51	13
Tiger	80	700	900	4	15.3	8	1.03	41	3.48	82	19
	80	750	884	6	15.0	10	1.74	53	3.02	70	15
Leopard	105	520	1041	4	17.4	12	1.05	48	2.90	74	21
	106	200	1040	1	15.0	7	1.69	40	2.33	60	20
Ocelot	104	885	1065	2	10.3	13	1.05	47	1.33	34	14
Sea-lion	104	885	745	2	1.0	7	1.53	41	1.04	28	19
	92	1020	140	3	—	—	1.51	38	1.00	39	10
Whale	I.M. 39	160	1047	4	22.3	7	1.35	37	2.74	60	18
	I.M. 30	160	1048	4	34.6	7	1.09	44	3.03	67	14

In considering the general nature of the haemopoietic process it is important to remember that although anti-anaemic preparations have been obtained from many animals, pernicious anaemia itself is unknown in any animal save man, and all attempts to produce it in experimental animals by such methods as gastrectomy have failed [cf. Morgan & Farrell, 1931; Ivy, Richter, Meyer & Greengard, 1934; 1933].

It is therefore evident that our knowledge of the biochemical and physiological processes involved in normal haemopoiesis and the deficiencies leading to the various anaemias is far from complete.

The haemopoietic process involves not only three chemical substances (the 'intrinsic' and 'extrinsic' factors of the stomach and the liver principle), but also a chain of physiological reactions, namely, the action of the 'intrinsic' and the 'extrinsic' factors in the stomach, the absorption of the products of this reaction (liver principle) from the intestine, the storage of the liver principle in the liver and its mobilization and transfer to the bone marrow when required. It is obvious that a break at any point in this chain may lead ultimately to the development of a megalocytic anaemia, which should normally be relieved or cured by replacement of the deficiency so produced. An exception, however, is seen in achrestic anaemia which follows the inability of the liver to release for use its stores of the haemopoietic principle [Wilkinson & Israëls, 1936].

In the present paper we are concerned solely with one link in the haemopoietic chain in mammals, namely, the presence of an anti-pernicious anaemia principle in the liver, and it has been demonstrated that in spite of species differences which are known to exist in the haemopoietic processes, most of the mammals investigated were able to store in the liver such an anti-anaemia principle which is effective in the treatment of pernicious anaemia. There is no evidence as yet to indicate whether the activities of these various extracts are due to the presence of one or more chemical substances.

SUMMARY

1. Extracts have been prepared from the livers of the following mammals:

Herbivorous: ox, sheep, horse, Indian elephant, African rhinoceros, Baringo giraffe, Nyloghaie antelope, gibbon, green monkey, red-faced Japanese monkey, chimpanzee, orang-utan.

Omnivorous and carnivorous: man, pig, lion, tiger, leopard, ocelot, Californian sea-lion, whale.

2. The yields of dry liver extracts were not constant and varied from 0.3 to 2.9 g./100 g. fresh-liver tissue.

3. All the extracts with the exception of those from the sea-lion livers have been shown to be clinically active in their ability to initiate remissions under controlled conditions in patients suffering from pernicious anaemia.

4. Thus, an anti-pernicious anaemia principle is stored in a large variety of mammalian livers.

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THE INFLUENCE OF ANTERIOR PITUITARY
EXTRACTS ON THE INSULIN CONTENT
OF THE PANCREAS OF THE HYPO-
PHYSECTOMIZED RAT

By MERVYN GRIFFITHS¹

From the National Institute for Medical Research, London, N.W. 3

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In 1933 Anselmino, Herold & Hoffmann claimed, on the basis of simple inspection of serial sections, that a few daily injections into normal rats of an extract of dried pituitary anterior lobe increased the amount of islet tissue in the pancreas. Richardson & Young [1937], using a quantitative method for the assay of islet tissue, failed to confirm this claim under the specified conditions and showed that the method of assessment by inspection was unsatisfactory. Nevertheless they were able to demonstrate a significant increase in islet tissue after 14-21 daily injections into rats of a saline extract of fresh anterior lobe of oxen. More recently Marks & Young [1939, 1940*a*] found that treatment with 7-14 daily injections of a crude extract of anterior lobe resulted in an increase in the insulin content of the normal rat pancreas to 130-250 % of that of the controls.

The question whether the anterior pituitary lobe secretes a 'pancreotropic' hormone, responsible for maintaining the islet tissue and insulin content of the pancreas, is obviously of great importance, yet as far as the writer is aware the only investigation concerned directly with this problem is that of Krichesky [1936] on the influence of hypophysectomy in the rat on the islets of Langerhans.² By the use of a quantitative method of assay of islet tissue Krichesky showed that hypophysectomy in the male Wistar rat increases the volume of the islet tissue, and that treatment of the hypophysectomized rat with a crude anterior lobe

¹ Science Research Scholar of the Royal Commission for the Exhibition of 1851. Present address: Department of Medicine, University of Sydney, N.S.W., Australia.

² See note at end by Dr F. G. Young.

extract can, to some extent, prevent this increase, a result which was, contrary to that expected from the hypothesis of a pituitary 'pancreo-tropic' hormone.

The present investigation was undertaken to determine the influence of hypophysectomy on the insulin content of the rat's pancreas, and the influence of different types of anterior pituitary extract on the pancreatic insulin of the hypophysectomized rat. As this work was nearing completion, Haist & Best [1940] published a note concerning the influence of diet on the insulin content of the pancreas of the hypophysectomized rat, in which they stated that in rats hypophysectomized for 26-66 days, pancreatic insulin was slightly lower than in control animals. The writer's experiments confirm this statement [cf. Griffiths & Young, 1940].

METHODS

Animals

Male Wistar-strain rats were used throughout. These were fed on a diet of powdered 'Purina fox chow' and water, and were kept at a temperature of 21° C. The duration of the period of hypophysectomy was from 2 to 5 weeks.

Assay of pancreatic insulin

The pancreases of groups of rats were bulked and extracted by a modification of the method of Jephcott [1932]. The potency of these extracts was estimated by the mouse method as carried out by Marks & Pak [1936].

Hypophysectomy

The animals were hypophysectomized by the retro-pharyngeal method of Selye [Collip, Selye & Thomson, 1933], under avertin anaesthesia. As the pancreases from a group of animals were necessarily bulked immediately after death for extraction of insulin, it was not feasible to determine the completeness of the removal of the gland of each animal of the group by cutting serial sections of the pituitary region, before its pancreas was added to the bulk. Other criteria of completeness had therefore to be rigidly applied, namely, cessation of growth, extreme atrophy of the adrenals and testes, and absence of macroscopic pituitary fragments in the sella turcica. In the case of those animals injected with pituitary extracts, these criteria, with the exception of inspection of the sella, could not be applied, so the absence of pituitary material had subsequently to be demonstrated in representative animals by serial sectioning of the pituitary region.

A control operation consisting of exposure of the pituitary was performed on some groups of animals, until it had been established, by comparison with intact animals, that pancreatic insulin was not significantly affected by the operation. Thereafter intact animals were used as controls. Both intact animals and those in which the pituitary gland had been thus exposed are referred to throughout as 'normal'.

Anterior pituitary extracts

A crude extract of ox anterior lobe was prepared by extracting absolutely fresh glands in the cold room with saline at pH 8.0-8.5, according to the method described by Young [1938]. Experiments in this laboratory have shown that this extract possesses growth-promoting, gonadotrophic, thyrotrophic, adrenotrophic, glycotropic, lactogenic, pancreotropic, and diabetogenic activities. As demonstrated by Marks & Young [1940a] such a crude extract loses its diabetogenic activity when incubated at 37° C. for 5 hr. Such a 'stale' crude extract was also used in some experiments in the present investigation. Some of the control animals were injected with saline or with an inert extract of thymus gland, prepared by the method for fresh gland extract.

Injectations were usually begun the day after the operation.

RESULTS

The results for groups of control animals showed that although the pancreatic insulin of the growing rat does not increase precisely in direct proportion to the body weight (Fig. 1), yet the relationship was sufficiently close to a direct one to justify a figure, calculated for the insulin content of the pancreas per 100 g. body weight, in comparing insulin contents of large groups of rats with different mean weights.

As the results seem to fall into two main classes—those for rats of about 80 g. weight, and those for rats of 100 g. or more—the data will be considered under these two headings.

80 g. rats. Hypophysectomy causes marked atrophy of the adrenals and testes, a significant decrease¹ in the weight of the pancreas from 0.67 to 0.44 g./100 g. body weight, increase in the insulin content of the pancreas from 0.68 to 1.06 units/100 g. (Table 1), and cessation of growth. These animals, however, do not lose weight as do those hypophysectomized at higher weights. Crooke [1938] has also observed that rats hypophysectomized at 80 g. do not markedly lose weight.

¹ Using Snedecor's 'F' test for analysis of variance. The writer is indebted to Dr Helen Turner, of the Macmaster Laboratory, Sydney, for this analysis.

Injection of fresh or 'stale' (non-diabetogenic) anterior pituitary extracts results in an increase in body weight and a coincident increase in pancreatic insulin. The insulin content per 100 g. body weight does not, however, remain at the high value characteristic of the 80 g. hypophysectomized rat (1.06 units/100 g. body weight) but now becomes similar to that for normal rats or for control hypophysectomized rats of body weight greater than 100 g. Thus when rats hypophysectomized

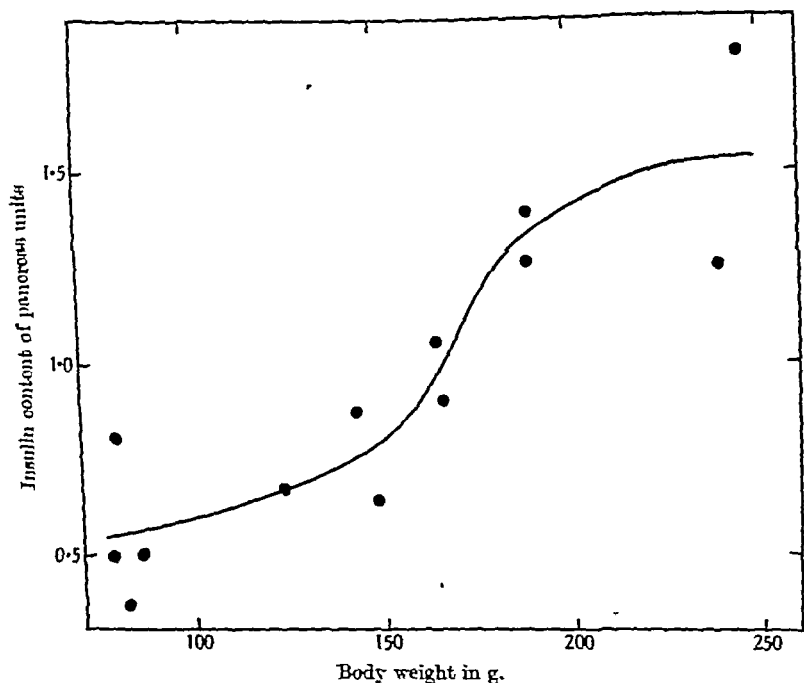


Fig. 1. The relationship between the total insulin content of the pancreas and the body weight of the Wistar-strain male albino rat.

at 80 g. had grown under the influence of anterior pituitary extract to an average weight of 177 g. the pancreatic insulin content was found to be 0.59 unit/100 g. (Table 1) a figure not significantly different from the values for normal rats (0.60 unit/100 g.) or for hypophysectomized rats of more than 100 g. body weight (0.71 unit/100 g., Table 2). The weight of the pancreas expressed in terms of g./100 g. body weight, is significantly lower in the treated hypophysectomized rats than in the normal controls.

Rats of 100 g. or more. As in rats operated on at 80 g. weight, hypophysectomy causes atrophy of the adrenals and testes, a significant decrease in the weight of the pancreas from 0.47 to 0.37 g./100 g. body

TABLE 1. Influence of hypophysectomy with and without treatment with anterior pituitary extract, on the insulin content of the 80 g. rat

Treatment	Days after operation	No. in group	Body weight g.		Insulin in pancreas units/100 g. rat	Pancreas weight g./100 g. rat	Testes weight g./100 g. rat	Adrenals weight mg./100 g. rat
			Initial	Final				
Pituitary exposed	—	20	—	80	1.00	0.70	1.13	26.2
Pituitary exposed	—	16	—	82	0.51	0.62	—	—
Intact	—	15	—	86	0.59	0.66	—	21.0
Intact	—	16	—	78	0.64	0.68	1.00	21.8
Average					0.68	0.67	1.06	23.0
Hypophysectomized	33	14	82	80	1.09	0.44	0.38	9.1
Hypophysectomized	28	13	86	81	1.20	0.43	0.26	8.1
Hypophysectomized	32	9	82	78	0.90	0.46	0.41	9.0
Average					1.06	0.44	0.35	8.7
Hypophysectomized 1 c.c. 1:1 fresh extract daily, for 26 days	29	11	83	190	0.45	0.42	0.43	8.6
Hypophysectomized 1 c.c. 1:1 'stale' extract daily, for 21 days	24	9	84	174	0.48	0.41	—	—
Hypophysectomized 1 c.c. 1:1 'stale' extract daily, for 21 days	24	10	83	167	0.85	0.41	0.57	10.6
Average					0.59	0.41	0.50	9.6

weight, but in these animals there is an average loss in body weight of 20 % of the initial value. The pancreatic insulin falls almost in proportion to the loss in weight so that the amount of insulin per 100 g. of rat is virtually the same as that for the normal controls (Table 2).

Injection of fresh and 'stale' anterior pituitary extracts again induces growth and an increase in pancreatic insulin which is approximately proportional to the increase in body weight. Thus the amounts of pancreatic insulin in the normal controls (0.60 unit/100 g.), the untreated hypophysectomized (0.54), and the treated hypophysectomized (0.71) are practically the same (Table 2). As with the 80 g. rats, the weight of the pancreas of the treated animals is significantly lower than that of the normal controls.

DISCUSSION

The results indicate that, in rats of 100 g. weight and over, the increase in pancreatic insulin which occurs during the period of growth is controlled by the pituitary gland; removal of the gland results in cessation of growth and of increase in pancreatic insulin. Replacement therapy by means of injection of pitu-

TABLE 2. Influence of hypophysectomy with and without treatment with anterior pituitary extract, on the insulin content of the rat weighing 100 g. or more

Treatment	Days after operation	No. in group	Body weight g.		Insulin in pancreas	Pancreas weight	Testes weight	Adrenals weight
			Initial	Final	units/100 g. rat	g./100 g. rat	g./100 g. rat	mg./100 g. rat
Pituitary exposed	—	19	—	124	0.55	0.52	1.35	19.3
Intact	—	10	—	144	0.61	0.55	—	—
Pituitary exposed	—	9	—	148	0.43	0.45	—	—
Pituitary exposed	—	7	—	166	0.63	0.56	—	—
Intact	—	12	—	167	0.54	0.47	1.35	16.0
Pituitary exposed	—	11	—	189	0.67	0.35	1.17	12.7
Pituitary exposed	—	8	—	189	0.73	0.44	1.23	14.5
Pituitary exposed	—	10	—	239	0.52	0.46	—	13.0
Intact	—	7	—	245	0.73	0.43	1.13	14.1
Average					0.60	0.47	1.25	14.9
Hypophysectomized	27	19	128	110	0.75	0.32	0.27	6.6
Hypophysectomized	28	19	160	121	0.65	0.32	—	—
Hypophysectomized	34	7	249	185	0.71	0.35	0.27	6.0
Hypophysectomized	28	8	101	82	0.73	0.47	—	—
Average					0.71	0.37	0.27	6.3
Hypophysectomized	27	19	128	110	0.75	0.32	0.27	6.6
Hypophysectomized 1 c.c. thymus extract daily, for 14 days	17	8	140	115	0.52	0.42	0.43	7.9
Hypophysectomized 1 c.c. saline daily, for 14 days	16	8	129	109	0.36	0.41	0.53	10.3
Average					0.54	0.40	0.41	8.2
Hypophysectomized 1 c.c. 1:8 'stale' extract daily, for 27 days	28	8	123	163	0.85	0.24	0.54	6.6
Hypophysectomized 1 c.c. 1:8 'stale' extract daily, for 16 days	17	10	126	150	0.59	0.37	1.11	8.0
Hypophysectomized 1 c.c. 1:1 fresh extract daily, for 16 days	18	16	124	191	0.71	0.43	0.83	11.0
Hypophysectomized 1 c.c. 1:1 'stale' extract daily, 5 or 14 days	18	11	127	170	0.70	0.40	0.99	11.2
Average					0.71	0.36	0.87	9.2

continuation of the normal insulin increase. However, there is no evidence that the increase of insulin can occur apart from the general increase in the constituents involved in somatic growth. In other words, there is no evidence that the pituitary secretes a specific insulin-increasing hormone. The results with rats hypophysectomized at 80 g. suggest that the

increase in insulin which is taking place in the growing normal animal of this weight, can occur in the absence of pituitary secretion. It is clear, however, that in both groups of hypophysectomized rats there is no deficiency in the amount of pancreatic insulin comparable with the atrophy of pancreas, adrenal and testis.

The fact that pituitary extracts increase insulin in the large normal and in the hypophysectomized rat, and increase the amount of islet tissue in large normal rats, together with the results of Haist & Best, shed no light on the apparent paradox, that Krichesky finds an increase in the volume of islet tissue in the hypophysectomized, large Wistar rat. It should be emphasized, however, that the cytology of either type of increase in size (that is, that due to the injection of pituitary extract and that due to hypophysectomy) is not known. It is probable that the hypertrophy described as induced in normal rats by anterior lobe extracts involves increase in the number of beta cells, but it is possible that the increase due to hypophysectomy is not accompanied by increase in the number and size of beta cells, but rather the hypertrophy and hyperplasia of some other elements. The observation of Adams & Ward [1936] that hypophysectomy in the newt, *Triturus viridens*, results in an increase in islet volume and in the number of 'pink' cells (considered by these authors to be the homologues of the alpha cells of mammals) and that no increase in the number of 'blue' cells (beta cells) occurs, supports this explanation.

It is obviously unwise to apply the results of these experiments to other species, as it has not yet proved possible to increase the insulin in the pancreas of the normal dog with the non-diabetogenic extracts which increase the insulin in normal rats [Marks & Young, 1940a]. Even the relationship between body weight and pancreatic insulin, observed in the rat, may not hold for other species, as it is difficult to detect insulin in the pancreas of the adult guinea-pig [Marks & Young, 1940b], this notwithstanding an abundance of beta cells in the islets of this species [Woerner, 1938].

SUMMARY

1. In the growing Wistar rat pancreatic insulin increases approximately in proportion to the increase in body weight.

2. In rats hypophysectomized at 80 g. body weight growth ceases without occurrence of loss in weight, pancreatic insulin is increased and atrophy of the pancreas occurs. In rats hypophysectomized at a body weight of 100 g. or more there is cessation of growth, approximately 20 % loss in body weight, atrophy of the pancreas, and a decrease

in its insulin content which is almost proportional to the loss in weight.

3. In both groups of rats injection of pituitary extracts causes increase in body weight due to the action of growth hormone and an increase in pancreatic insulin simultaneously with the increase in body weight. This evidence indicates that although the pituitary gland controls the increase in pancreatic insulin during growth, it may not do so by means of a specific insulin-increasing hormone, but rather the increase is associated with the general increase in constituents involved in somatic growth.

It is with pleasure that the writer acknowledges his indebtedness to the Director of the National Institute for Medical Research, Sir Henry Dale, for his advice and extension of laboratory facilities, and to Dr F. G. Young of the same institute, who suggested this investigation, for his advice.

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Note added by F. G. Young 20 February 1941. Owing to war conditions, Mr Griffiths was clearly not aware of the paper by H. Y. Soong in the issue of the *Chinese Journal of Physiology* dated June 1940, when he sent this paper from Australia for publication in Great Britain. Soong (loc. cit. 15, 335) has also examined the influence of anterior pituitary extract on the insulin content of the normal and of the hypophysectomized rat, and his results and those of Griffiths agree in general burden though not entirely in detail. A discussion of these differences must await a future publication.

THE ANTAGONISM BETWEEN INSULIN AND
POSTERIOR LOBE PITUITARY EXTRACTBY MERVYN GRIFFITHS¹*From the National Institute for Medical Research, N.W. 3**(Received 10 February 1941).*

THE experiments of Burn [1923] were among the first to demonstrate clearly that posterior lobe pituitary extracts can inhibit the hypoglycaemia due to subcutaneous injection of insulin. Numerous reports [e.g. La Barre, 1937; Van Dyke, 1936, 1939] have since confirmed this finding but relatively little attention has been paid to the problem of the effect of posterior lobe extract on the hypoglycaemia due to intravenous administration of insulin. Geiling, Campbell & Ishikawa [1927], and Geiling, de Lawder & Rosenfeld [1931] found that, in dogs, whole posterior lobe extract and its separated oxytocic and pressor fractions inhibit hypoglycaemia due to intravenously administered insulin. Ellsworth [1935] concludes, however, that only the oxytocic fraction is active in the dog, and that any such activity of the pressor fraction is due to contamination with oxytocic substance. It has been pointed out [Lambie & Redhead, 1929] that the antagonism between posterior lobe extract and insulin may not be of a direct nature, but that the profound circulatory changes produced by the pituitary extract may modify the rate of diffusion into the tissues of injected material. A decreased rate of absorption of subcutaneously administered insulin, due to circulatory changes in the skin, may account in part for the inhibitory action of the pituitary extract [Young, 1938].

This investigation was undertaken with the object of obtaining further data on the action of posterior pituitary extract on intravenously administered insulin. In addition, it was considered that comparison of the effects of this extract on intravenously and on subcutaneously injected insulin, in the same animal, might yield data relevant to the question of the existence of such modes of action as suggested by Lambie & Redhead, and by Young.

¹ Science Research Scholar of the Royal Commission for the Exhibition of 1851. Present address: Department of Medicine, University of Sydney, N.S.W., Australia.

METHODS

Animals. The animals used in all the experiments were unanaesthetized, inbred, male, Dutch rabbits, weighing 1.5–2.0 kg. They received an ample diet of cabbage, bran, oats and hay.

Injections. After a fast of 21 hr. 3 units of crystalline insulin (Burroughs Wellcome) were administered either subcutaneously or intravenously, and, at the same time, by the subcutaneous route, 40 units (4 c.c.) of Burroughs Wellcome posterior lobe extract (Infundin) were given. In two experiments 10 units of insulin were injected intravenously and the posterior lobe extract was administered subcutaneously 65 min. afterwards. Control injections of 3 units of insulin and 4 c.c. of 0.25 % acetic acid were made on each fasting rabbit, and a few days later the effect of simultaneously administered insulin and infundin was determined on the same rabbit.

Blood-sugar estimations. Venous blood samples were taken 5, 10, 20, 30, 40, 60, 90, 120, 150 and 180 min. after the injection of insulin. Blood sugar was determined on 0.1 c.c. of blood by the method of Hagedorn & Jensen.

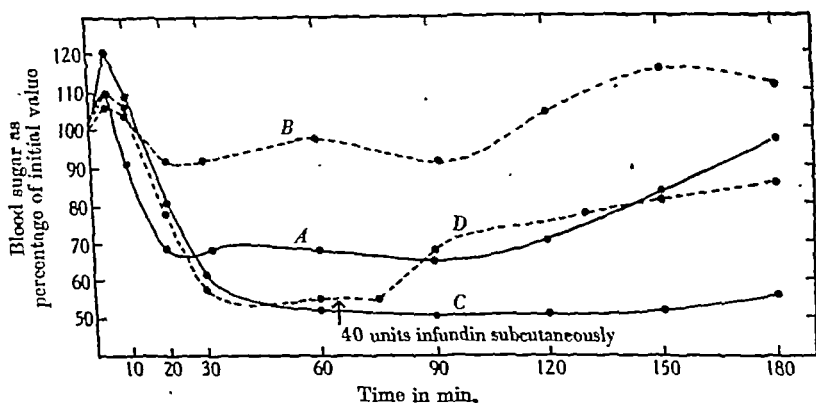


Fig. 1. Rabbit 14. A. Response to intravenous injection of 3 units of insulin and simultaneously 4 c.c. 0.25 % acetic acid, subcutaneously. B. Response to intravenous injection of 3 units of insulin and subcutaneous injection of 40 units of infundin, simultaneously. C. Response to intravenous injection of 10 units of insulin. D. Response to intravenous injection of 10 units of insulin, followed 65 min. afterwards by a subcutaneous injection of 40 units of infundin.

RESULTS

With intravenously injected insulin

The effect of infundin on the hypoglycaemia due to intravenously administered insulin varied from rabbit to rabbit, but the results for

individual rabbits were constant. In some rabbits the pituitary extract inhibited the hypoglycaemic action of the insulin (Fig. 1, curve *B*) and in others it was without effect (Fig. 2, curve *B*). In one of the experiments,

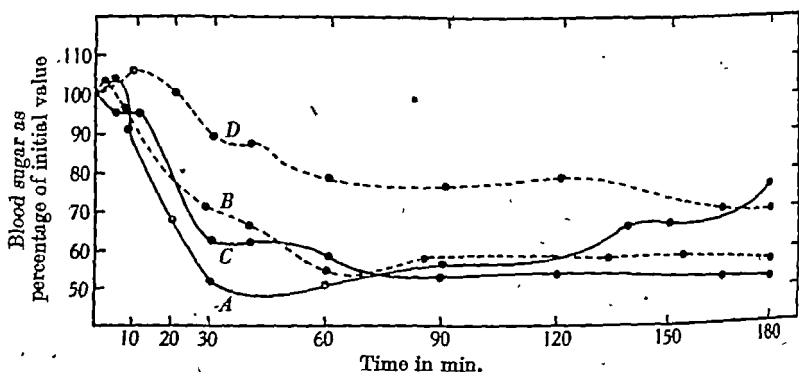


Fig. 2. Rabbit 544. *A*. Response to intravenous injection of 3 units of insulin and simultaneous subcutaneous injection of 4 c.c. 0.25% acetic acid. *B*. Response to intravenous injection of 3 units of insulin and simultaneous subcutaneous injection of 40 units of infundin. *C*. Response to subcutaneous injection of 3 units of insulin and simultaneous subcutaneous injection of 4 c.c. 0.25% acetic acid. *D*. Response to subcutaneous injection of 3 units of insulin and simultaneous subcutaneous injection of 40 units of infundin.

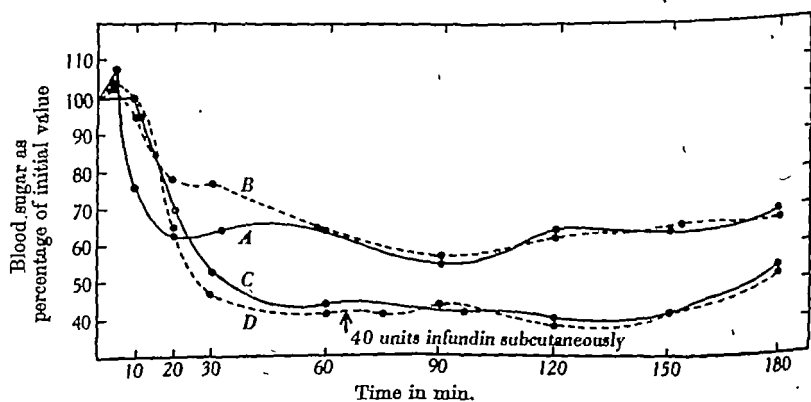


Fig. 3. Rabbit 13. *A*. Response to intravenous injection of 3 units of insulin. *B*. Response to intravenous injection of 3 units of insulin and simultaneous subcutaneous injection of 40 units of infundin. *C*. Response to intravenous injection of 10 units of insulin. *D*. Response to intravenous injection of 10 units of insulin followed 65 min. later by a subcutaneous injection of 40 units of infundin.

in which pituitary extract was given 65 min. after the injection of 10 units of insulin, the infundin was markedly effective in raising the blood sugar from the low level to which it had fallen (Fig. 1, curve *D*). In a similar

experiment on another rabbit infundin was completely without effect (Fig. 3, curve *D*), as was the case in this animal with simultaneously administered insulin and infundin (Fig. 3, curve *B*).

With subcutaneously administered insulin

Invariably in a series of eight rabbits, the pituitary extract inhibited the action of the insulin. Curve *D* in Fig. 2 illustrates a typical example of the effect. There was no effect on the hypoglycaemia due to intravenous insulin in this animal, nor in another of the series.

DISCUSSION

The data from those experiments in which the pituitary extract inhibited the action of subcutaneously administered insulin, but failed to inhibit that of intravenously administered insulin, constitute strong evidence in support of the suggestion that the inhibitory effect of pituitary extract on the action of subcutaneous insulin is due to depression of the rate of absorption. It seems reasonable to assume that this is due to the vaso-constrictor action of posterior lobe extract. However, it is quite apparent that infundin can antagonize intravenously injected insulin in a limited number of individual rabbits. The findings of Magenta [1929] that small doses of the non-pressor oxytocic fraction can inhibit, in the dog, the hypoglycaemia due to injection of subcutaneous insulin, and of Ellsworth [1935] that very small doses of the same fraction can inhibit the action of intravenously administered insulin, indicate that probably the antagonism is of a direct nature, in which circulatory changes play little part. However, many have claimed (see La Barre) that in other species, including man [Schroeder, 1933], the oxytocic fraction fails to antagonize subcutaneously administered insulin, and that the pressor fraction has marked activity. This may mean that direct insulin antagonism is absent in these species and that the inhibition with the pressor fraction is due to delayed absorption of insulin. The effect of each of the separate fractions on intravenously injected insulin in these species must be studied before anything more definite can be stated as to the nature of the antagonism. It is obvious, however, from the writer's results that subcutaneous injections of insulin are unsuitable for the study of this antagonism.

SUMMARY

In the rabbit posterior pituitary extract invariably inhibits the hypoglycaemia due to the subcutaneous injection of insulin, but in many of the rabbits used, no antagonism to intravenously administered insulin

was found. In other rabbits the antagonism was well defined. This suggests that the antagonism between subcutaneously administered insulin and posterior lobe extract is mainly due to a decreased rate of absorption, probably as the result of the vaso-constrictor action of the pressor substance.

No satisfactory answer can yet be given to the question of the existence of a direct antagonism between insulin and posterior lobe extract.

It is with pleasure that the writer acknowledges his indebtedness to the Director of the National Institute for Medical Research, Sir Henry Dale, for his advice and extension of laboratory facilities, and to Dr F. G. Young of the same institute for his advice.

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ON THE PERMEABILITY CHANGE OF STIMULATED NERVE

BY J. F. DANIELLI

(*Beit Memorial Research Fellow*)

From the Sir William Dunn Institute of Biochemistry, Cambridge

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For many years the view has been held that the response of a nerve to a stimulus consists initially of a local increase in permeability of the nerve membrane, which permits of the discharge of neighbouring regions of the membrane. This discharge is supposed to increase the permeability of these regions also, giving rise by this means to a wave of discharge and increased permeability which travel down the nerve. This view has been summarized by R. S. Lillie [1923].

Recent observations by K. S. Cole [personal communication] have shown that in *Nitella* and in squid nerve the passage of the impulse is accompanied by a sudden decline in impedance of the cell membrane, which is almost entirely due to a decrease in the ohmic resistance, the capacitance being almost unchanged, i.e. the change in permeability postulated by Lillie does in fact occur. In view of this it seems profitable to suggest a molecular mechanism by which this change of impedance (or permeability) may occur. The present hypothesis is based on Langmuir's [1933] theory of the structure of expanded films. It was, however, largely stimulated by Hill's [1932] demonstration that the heat evolution accompanying the passage of a single impulse is much smaller than the free surface energy of an oil-water interface, and by Hill's comment that this fact made it unlikely that complete breakdown of a surface film could be involved.

The cell membrane almost certainly consists of a thin film of lipoid material, about 50 Å. thick, with a layer of protein adsorbed on to both surfaces, and we shall assume that the nerve membrane is two molecules thick [Harvey & Danielli, 1938]. The change of permeability must be due to a change in the structure of this film. With the 'resting' nerve there is

a potential across this film of about 50 mV. (the resting or 'injury' potential), and whilst under this potential the film has a low permeability. When the potential is discharged the permeability is increased. The potential gradient across the film due to the injury potential cannot be less than $0.05 \text{ V./}5 \cdot 10^{-7} \text{ cm.}$, i.e. $100,000 \text{ V./cm.}$ This is the right order of magnitude to produce a marked orienting effect on molecules having polar groups such as C.OH, C.NH₂, etc. [see, e.g., Malsch, 1929; Debye, 1935]. The potential may be as high as 10^7 V./cm. if the potential drop of 50 mV. takes place over a part only of the thickness of the film.

From the work of Langmuir, Adam, Rideal and their colleagues, a great deal of information is available on the structure of monolayers of fatty materials, but no work has been done on the effect of a potential applied perpendicular to the plane of the monolayer.¹

However, the molecules forming a monolayer are known to be oriented more or less perpendicular to the plane of the film. Any change in structure due to an applied potential must be due to a reorientation of molecules. This reorientation in the nerve membrane, due to the resting potential, must be such as to reduce the permeability to ions: when the potential is discharged, as in 'electrical stimulation of nerve, the change in orientation must involve an increase in permeability.

NATURE OF THE PERMEABILITY CHANGE

Any monolayer in which polar molecules are closely packed together should show molecular orientation and consequently, owing to the orientation of the electrical dipoles of the polar groups, there is a potential difference, E_s , between the two sides of the monolayer, which can be measured in many cases [see Adam, 1938, for a review]. The electrostatic field in the surface has a gradient of the order of 10^6 V./cm. or more, in the vicinity of the polar groups. An applied potential of appropriate direction and magnitude will act upon the electrostatic dipoles of the oriented molecules so as to cause reorientation. But most monolayers (e.g. those of sterols) are already oriented in the surface field in a gradient of the order of 10^6 V./cm. , are insensitive to temperature changes, and an applied gradient of $100,000 \text{ V./cm.}$ could not be expected to make much difference to the molecular packing. One class of monolayers, the expanded films, which includes such substances

¹ The present writer, in collaboration with Dr W. A. H. Rushton, has made an attempt to carry out such experiments on monolayers at an air-water interface, but it has been found impossible to obtain sufficiently high gradients, sparks crossing the gap between the electrode and the film at a potential gradient of less than 5000 V./cm.

as lecithin and the glycerides, is an exception to this rule. At sufficiently low temperatures the molecules of these monolayers are closely packed together [Adam & Jessop, 1926], forming films which must be of very low permeability to ions, since they consist of tightly packed fatty molecules between which ions cannot dissolve. At sufficiently high temperatures the molecules are very loosely packed, forming a two-dimensional gas. Over an intermediate range of temperature, about 10 to 40° C., the monolayers are composed of a mixture of small micelles of the close-packed film and of more loosely packed molecules: in the loosely packed regions the adhesions between the hydrocarbon parts of the molecules have been partly, but not completely, broken down [Langmuir & Schaeffer, 1936]. The average area per molecule in the close-packed micelles will be, e.g., 25 sq. Å., and in the loosely packed part 40–50 sq. Å. The effect of raising the temperature is to increase the proportion of molecules in the loosely packed condition. This means that if the area of the whole monolayer is kept constant, the average area per molecule in the loosely packed areas is reduced. Thus at, say, 10° C. a monolayer¹ may consist of 50% of micelles having an average area of 25 sq. Å. per molecule, and 50% of loosely packed molecules, occupying an average area of 40 sq. Å. per molecule. At 20° C. the same monolayer, if occupying the same total area as at 10° C., will consist entirely of molecules in the expanded state, occupying an average area of 32.5 sq. Å., and the micelles will be abolished. Thus, with such a monolayer, the reorientation due to a rise in temperature of 10° C. is sufficient to reduce the area per molecule in the most loosely packed areas from about 40 to 32.5 sq. Å. The closely packed micelles will be very difficult indeed for ions to penetrate. The surfaces even of liquid hydrocarbons are very resistant to penetration by ions, as is shown by the low solubility of salts in hydrocarbons, and by the low electrical conductivity of thin hydrocarbon layers placed between salt solutions. The micelles have a closer, more rigorously restricted structure than an isotropic liquid hydrocarbon, and should be even less permeable to ions.

The loosely packed regions of the monolayer fall into another category. The average area per molecule is far more than the minimum area (20 sq. Å. [Adam, 1938]) into which such molecules can be packed, so that we can expect that when the average area per molecule in the loosely packed areas is 40 or 50 sq. Å., these regions should be fairly permeable to ions. On the other hand, as the area per molecule approaches that of the micelles, ionic permeability should decrease rapidly.

¹ E.g. of myristic acid.

That the permeability of the loosely packed areas of these films is proportional to the average area per molecule is an hypothesis which cannot be tested experimentally at present, since no method has been found of obtaining such a film between two aqueous phases. It is introduced here as a reasonable working hypothesis.

A potential applied across an expanded monolayer, increasing the surface potential E_s , will tend to give molecules in the micelles a different orientation and consequently must reduce the degree of micelle formation, since this depends on a unique orientation of the molecules:¹ it will therefore decrease the average area per molecule in the intermicellar regions. Its effect should thus be similar to that of raising the temperature, and will make the monolayer less permeable to ions. It follows that if the nerve membrane has the structure of an expanded film the effect of the resting potential is to maintain a low permeability, and a decrease of the injury potential will produce an increase in permeability. Hence, with such a structure, excitation will occur at the cathode on 'making' an electrical circuit. On breaking the circuit there will be a decrease in permeability at the cathode, and hence no excitation. Before considering this in detail it will be profitable to discuss the heat production in micelle formation.

HEAT EVOLVED DURING PASSAGE OF THE IMPULSE (INITIAL HEAT)

According to the suggested mechanism, the impulse consists essentially of two factors: (1) a change in permeability of the membrane due to condensation of the monolayer into micelles, which is later reversed as the impulse passes down the nerve; (2) a flow of ions across the membrane, which involves mixing of K^+ ions with plasma, serum or Ringer, and the mixing of Na^+ ions with the cell contents. The formation of micelles involves liberation of a certain amount of heat H_m [Langmuir, 1933], and the subsequent evaporation in a return to the initial state involves absorption of the same amount of heat. In the period covered by the initial heat production of nerve, the membrane molecules will pass through a nearly complete cycle of micelle formation and evaporation, so that any heat evolution due to the permeability change should be only a fraction, say not more than 10%, of H_m .²

The mixing of Na^+ and K^+ solutions, due to passage of these ions across the nerve membrane, will also result in an evolution of heat. Call

¹ The vertical component of the electrical dipole of the molecule is less in the expanded state than in the micelles.

² It will probably be much less than this.

this H_i . Then the total heat evolved during passage of an impulse is not greater than $(H_m/10 + H_i)$; i.e. with the mechanism suggested here, the initial heat of nerve must be not greater than $(H_m/10 + H_i)$. If the initial heat were greater than this, it would be a certain indication that an important factor has been neglected, and the mechanism proposed here could not be correct. If the initial heat is less than $(H_m/10 + H_i)$, but not less than H_i , then the proposed mechanism is a possible one. If the initial heat is less than H_i , the theory proposed here must be wrong in at least one important factor. We must now calculate H_m and H_i .

VALUE OF H_m

As we have no knowledge of the details of the molecules composing the nerve plasma membrane, we cannot calculate the heat evolved by condensation into micelles for a nerve membrane. We can only take a typical case, such as that of myristic acid films, and calculate H_m for this monolayer, thus obtaining the order of magnitude of H_m . According to Langmuir [1933], the latent heat of this change is 61.6×10^{-14} ergs per molecule. If, as in the example we are dealing with, the average area per molecule is 32.5 sq. Å., the total number of molecules per sq. cm. is $10^{16} \div 32.5$. And if half of these molecules condense into micelles, the heat evolved will be

$$\begin{aligned} H_m &= \frac{1}{2} \times \frac{10^{16}}{32.5} \times 61.6 \times 10^{-14} \text{ ergs/sq. cm.} \\ &= 95 \text{ ergs/sq. cm.} \end{aligned}$$

For stearic acid the value would be 105 ergs/sq. cm.

CALCULATION OF H_i

According to Cowan [1934] one impulse allows 2×10^{-13} equivalents of K^+ to flow from 1 sq. cm. of crab's nerve surface. An equivalent amount of Na^+ probably enters the nerve, so that we can place 5×10^{-13} equivalents as an upper limit to the total ion flow both into and out of the nerve, per impulse. Judging from the heats of dilution of K^+ and Na^+ solutions [see, e.g., Taylor, 1930], the heat evolved on mixing isotonic solutions containing these ions will not exceed 10 cal./g. equivalent, at the concentrations found in crab's nerve. For safety, let us assume that in the case of both the Na^+ and the K^+ ions there will be a heat evolution of this magnitude. Actually there will probably be a heat evolution due to the dilution of one ion and a heat absorption due to dilution of the other, but if we assume both heats are positive we obtain a maximum value for H_i , eliminating any possibility of error due to underestimating

this heat evolution. Then the heat evolved due to ions flowing across the membrane is

$$H_1 = 5 \times 10^{-13} \times 10 = 5 \times 10^{-12} \text{ cal./sq. cm.} \\ = 2 \times 10^{-4} \text{ ergs/sq. cm.}$$

We thus find that the initial heat evolved by the suggested mechanism must not be greater than $(\frac{95}{10} + 2 \times 10^{-4})$, or 9.5 ergs/sq. cm. of nerve axon surface, and not less than 2×10^{-4} ergs/sq. cm. The value of the initial heat for crab's nerve is 2×10^{-2} ergs/sq. cm. [Hill, 1932]: for frog's nerve the initial heat is between 5×10^{-3} and 2.5×10^{-4} ergs/sq. cm., according to the experimental conditions. We may thus conclude that the initial heat of nerve falls within the limits demanded by the theory presented here.

DETAILS OF MEMBRANE STRUCTURE

Since the energy relationships are satisfactory, we may consider the structure of the postulated membrane in a little more detail. Fig. 1

CONVENTIONS

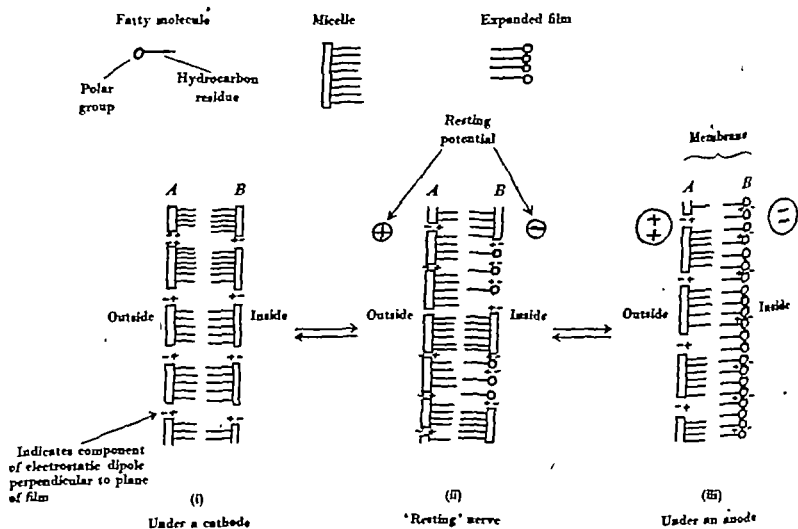


Fig. 1.

shows the postulated structure in three different conditions, the two layers of fatty molecules being labelled A and B respectively: (i) is the film corresponding to a discharged nerve membrane, i.e. in the region under a cathode; it consists almost entirely of micelles with gaps between

tem, the gaps being large enough for ions to penetrate: (ii) corresponds to a 'resting' nerve; the positive and negative signs inside circles indicate the direction of the resting potential, and the signs on the molecules of the films indicate a component of the electrostatic dipoles of the film molecules which is perpendicular to the plane of the film. The resting potential is in the opposite direction to the surface potential of layer *A* of the film; consequently the structure of this layer, and therefore its permeability, are practically unchanged, compared with state (i). But the dipoles of layer *B* are reoriented; this layer partly passes into the gaseous state, and the permeability of the film is decreased due to partial filling of the holes between micelles: (iii) is the state of the film when the potential across it is increased above the value of the injury potential, as is the case beneath an anode. Layer *A* is again unchanged, but layer *B* becomes still less permeable. Consequently, at an anode, stimulation cannot occur on 'making' a circuit. If the anodic potential is maintained for a sufficient period the increase in surface pressure in layer *B* (due to transformation to an expanded film) will cause molecules of the film to move down the film away from the anode. Thus, when the anodic potential is removed, there will be fewer molecules in layer *B* under the electrode than are necessary to maintain normal permeability. Hence, on breaking a circuit, an increase in permeability will occur under an anode which will exceed that due to the simple change from condition (iii) to condition (ii). If the excess increase in permeability is sufficiently large, excitation will occur.

With this structure, the process of accommodation [Hill, 1936] must include the movement of ions through the film, and molecular rearrangement in the film.

The effect of adding potassium to Ringer is to decrease the injury potential. Hence the ionic permeability is increased, and the process of accommodation should be accelerated. The action of calcium ions on films is to react with the acidic groups, reducing the tendency to form gaseous films and increasing the amount of close-packed film [Langmuir & Schaefer, 1936]. Hence adding Ca should also increase ionic permeability, and accelerate accommodation. Moreover, since accommodation involves the slow process of leakage of ions through a film, it should have a high Q_{10} [Danielli, 1937]. Solandt [1936] found that Ca and K both accelerate accommodation and that accommodation has a Q_{10} of about 3.

This simple theory is adequate to explain qualitatively the observed behaviour of muscle and nerve. The great weakness is that it is difficult to predict the time relationships of the various postulated processes.

Until these time relationships can be deduced, methods of testing the theory seem to be restricted to impedance studies of the action of ions and temperature change on the nerve membrane. These will show whether the details of the predicted permeability changes are correct, from a qualitative point of view.

The present theory is not proposed as an alternative to Lillie's theory. It is intended to provide a physical basis for the permeability change which forms an essential part of Lillie's theory. All the various processes accompanying the passage of an impulse, such as, e.g., the restoration of the resting structure of the membrane, are assumed to occur just as Lillie suggested, by the intervention of local electrical currents.

SUMMARY

It is suggested that the lipid part of plasma membrane of nerve axons is a bimolecular film, composed of two partly expanded monolayers, that the effect of the injury potential is to increase the degree of expansion, and that the increase of permeability found on discharging the injury potential is due to a reversion to close-packed film with gaps permeable to ions between the areas or micelles of close-packed molecules. It is shown that with this structure the initial heat of nerve should lie between the approximate limits of 10 and 2×10^{-4} ergs/sq. cm. of nerve surface; that excitation will occur at 'make' at the cathode and at 'break' at the anode; that K^+ and Ca^{++} should both increase the rate of accommodation, and that accommodation should have a high Q_{10} .

I am indebted to Mr A. L. Hodgkin and Prof. A. V. Hill for their kind criticism and advice.

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DIFFERENTIATION OF AN (AMPHIBIAN) WATER BALANCE PRINCIPLE FROM THE ANTIDIURETIC PRINCIPLE OF THE POSTERIOR PITUITARY GLAND

By H. HELLER (*Beit Memorial Research Fellow*)

From the Department of Pharmacology, Oxford

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An injection of mammalian posterior pituitary extract into an amphibian kept in water causes a gain of weight due to a temporary increase of the animal's body water. The smallest dose which produces a significant increase of weight in frogs (*Rana esculenta*) is approximately 0.1 unit or 100 milliunits (mU.) of a posterior pituitary preparation [Heller, 1930a]. (Boyd & Brown [1938], working with American frogs (*Rana pipiens*), obtained a comparable figure.) It has recently been shown [Heller, 1941a] that the pituitary gland of the frog contains an antidiuretic activity equivalent to only about 3.5 mU. pitressin. In view of the discrepancy between the small amount of antidiuretic principle found in the frog pituitary gland and the minimum amount of a (mammalian) posterior pituitary extract needed to produce an increase of body water it looks as though the antidiuretic hormone had little significance for the regulation of the water balance of amphibians.

Extracts of frog pituitary glands were therefore prepared and their action on the water metabolism of frogs tested.

METHODS

Frog assay of pituitary extracts. English frogs (*R. temporaria*) of an average weight of 20 g. were used. All experiments were done between March and October. The evening before the experiment started each frog was placed in a numbered beaker and immersed in tap water. The beakers were covered with glass plates to prevent undue losses of weight due to air currents. Changes of weight of frogs kept under these conditions at room temperature were small and inconsistent. For example, the mean changes of weight in 48 hr. of a series of fifty frogs were found to be

$-2.2 \pm 2.84\%$. Cross tests were used to compare the effect of any preparations on the water uptake of a series of frogs. As a rule five of a series of ten frogs were injected with one preparation, the other five with the other extract. After an interval of 48 hr. the groups were changed round and the animals injected with the preparation which had not been administered in the first experiment. Injections were made into a ventral lymph sac. The volume of each injection was uniformly 0.5 c.c. Weighings were made 1 hr. before the injection and subsequently at hourly or half-hourly intervals. Before each weighing the frogs were carefully dried and the bladder thoroughly emptied.

Oxytocic and pressor assay. Guinea pigs' uteri and spinal cats were used.

Preparation of pituitary extracts. With the exception of the fish pituitary glands which were obtained from dead fishes kept in a refrigerator, glands of freshly killed animals were used. If not otherwise stated (see p. 135) extracts were prepared from the whole pituitary gland including the pars tuberalis and the tuber cinereum. For details of extract preparation see a previous paper [Heller, 1941a]. Control extract of pieces of indifferent brain tissue of the various species of vertebrates used were prepared in an identical manner, but had no significant effect on the water uptake of frogs.

The commercial (mammalian) post-pituitary extracts employed were Messrs Parke, Davis & Co.'s pituitrin, pitocin and 'specially prepared' pitressin and B.D.H. posterior pituitary extract.

RESULTS

Is the 'water balance' principle identical with any of the known hormones of the posterior pituitary gland?

It will be noted (Figs. 1-3) that the weight of a series of frogs each injected with the extract of one frog pituitary gland increases for over 5 hr., reaching a maximum of over 10% above the initial weight. It was mentioned above that the antidiuretic activity of one frog pituitary gland equals about 3.5 mU. pitressin [Heller, 1941a]. Fig. 1 shows that the injection of 10 mU. of pitressin per frog has practically no effect on the water balance. An increase of the duration and intensity caused by the extract of a single frog pituitary gland can, therefore, hardly be explained by its content of antidiuretic activity. The dose of a commercial (mammalian) post-pituitary extract which has to be injected to obtain an effect comparable to that of the extract of a single frog pituitary gland, amounts to about 800 mU. (Fig. 2).

The objection could be raised that the action of commercial post-pituitary extracts is modified by the more complicated methods used for its preparation. Rat pituitary extracts, prepared in the same manner as

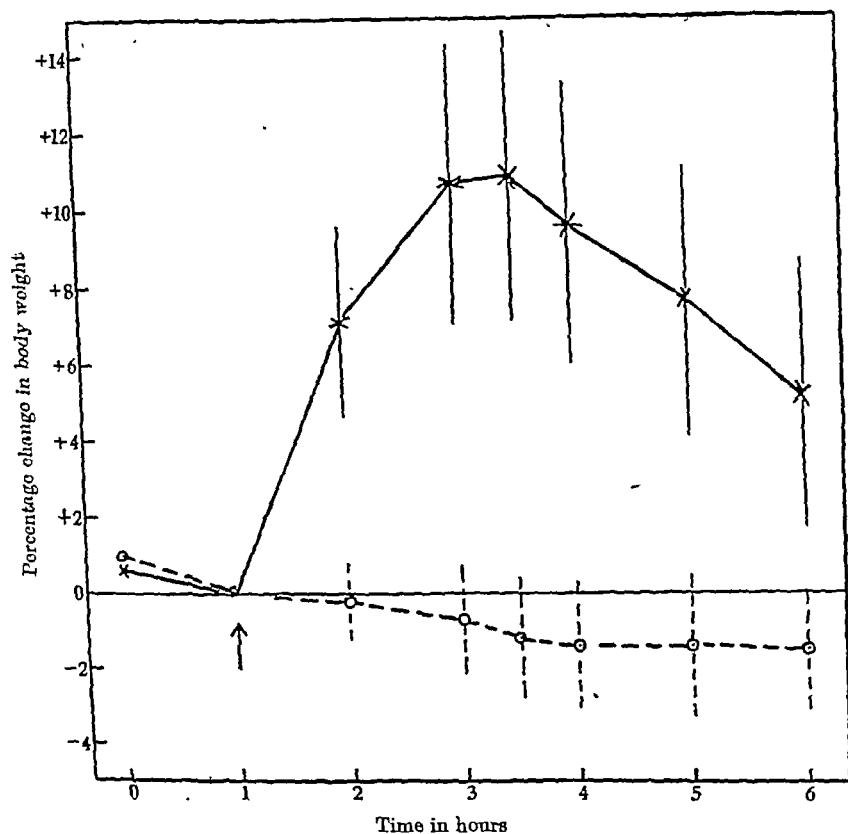


Fig. 1. The difference between the effect on the water balance of frogs of the extract of a single frog pituitary gland and of 10 mU. pitressin. Note that the maximum anti-diuretic activity of a frog pituitary gland was found to equal 5 mU. pitressin. X—X mean percentage changes in weight of twenty frogs injected with the extract of one frog pituitary gland each. O—O same frogs injected with 10 mU. pitressin each. Injections at the time marked by arrow. The vertical lines indicate the standard error. In this and the following experiments the significance of the differences observed was investigated by Fisher's 't' test. The following figures were obtained for the present experiment: t (for maximum increases of weight) = 2.76, $P < 0.02$.

the frog pituitary extracts, were therefore assayed for their effect on the frog's water balance. Table 1 shows that the extract of one rat pituitary gland, which contains about 1000 mU. of the antidiuretic hormone [Heller, 1941a], causes a smaller increase of the body weight of frogs than

the extract of one frog pituitary gland which contains the equivalent of 3.5. It seems therefore justifiable to assume that the principle causing the changes of the body water of frogs (water balance principle) and the

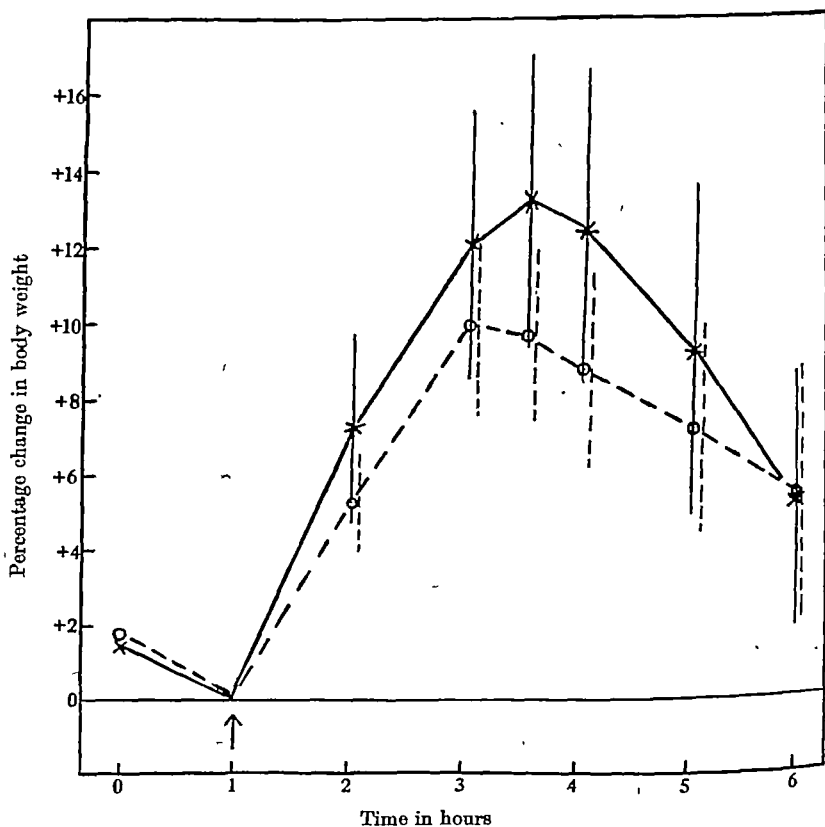


Fig. 2. The dose of a commercial (mammalian) posterior pituitary extract required to equal the water-balance activity of an extract of a single frog pituitary gland. X—X mean percentage changes in weight of twenty frogs injected with the extract of one frog pituitary gland each. O—O same frogs injected with 800 mU. of B.D.H. posterior pituitary extract each. Injections at the time marked by arrow. The vertical lines indicate the standard error. The values for standard errors indicated by broken lines and belonging to the graph indicated by broken lines were obtained at the same relative times as those shown in full lines but are placed alongside for technical reasons. t (for maximum increases of weight) = 0.73, $P < 0.5$.

post-pituitary antidiuretic hormone are not identical. This conclusion agrees with earlier results [Heller, 1930*b*; Steggerda & Essex, 1934, and others], which proved that the vasopressor-antidiuretic fraction of post-

TABLE 1. Comparison between the effect of *frog* and of *rat* pituitary extracts on the water balance of frogs. The extract of one *rat* pituitary gland which contains about 1075 mU. of the antidiuretic activity causes a smaller average increase of the body weight than the extract of one *frog* pituitary gland which contains about 3.5 mU. *Series A*=ten frogs injected with extract of one *rat* pituitary gland each. *Series B*=ten frogs injected with extract of one *frog* pituitary gland each. Weight immediately after injection = 100%, *t* (for maximum weight increases) = 3.34, $P < 0.01$

Time after injection (hr.)	<i>Series A</i> Percentage changes of body weight	<i>Series B</i> Percentage changes of body weight
1.0	$\div 3.3 \pm 1.33$	-7.5 ± 2.51
2.0	$\div 4.4 \pm 1.05$	$\div 11.6 \pm 3.47$
2.5	$\div 4.2 \pm 0.73$	$\div 12.6 \pm 2.19$
3.0	$\div 3.6 \pm 0.94$	$\div 12.6 \pm 2.03$
4.0	$\div 2.9 \pm 1.13$	$\div 10.9 \pm 2.55$
5.0	$\div 2.1 \pm 0.86$	-8.3 ± 2.39
24.0	-0.7 ± 2.24	-0.5 ± 1.80

pituitary extracts had a considerably weaker action on the water uptake of frogs than the oxytocic fraction.

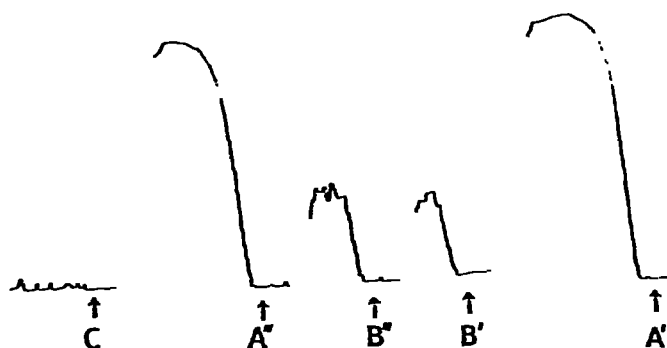


Fig. 3. The oxytocic activity of an extract of one frog pituitary gland. Isolated uteruses of guinea-pig. Read from right to left. A, A'=8 mU. posterior pituitary extract B.D.H. B, B'=20 volume % of extract of one frog pituitary gland. C=control extract of indifferent frog brain. The extract of one frog pituitary gland contains less than 40 mU. of the post-pituitary oxytocic principle.

This suggests the possibility of an identity of the oxytocic hormone with the amphibian water-balance principle. The oxytocic potency of frog pituitary extracts was, therefore, determined. Fig. 3 establishes it at considerably less than 40 mU. (oxytocic) per one frog pituitary gland. These results suggest strongly that the effect on the water balance of

frogs exerted by the extract of frog pituitary glands is not due to their oxytocic activity. The difference between the action of an injection of 40 mU. of pitocin per frog and the effect of an injection of the extract of one frog pituitary gland per frog is clearly shown in Fig. 4.

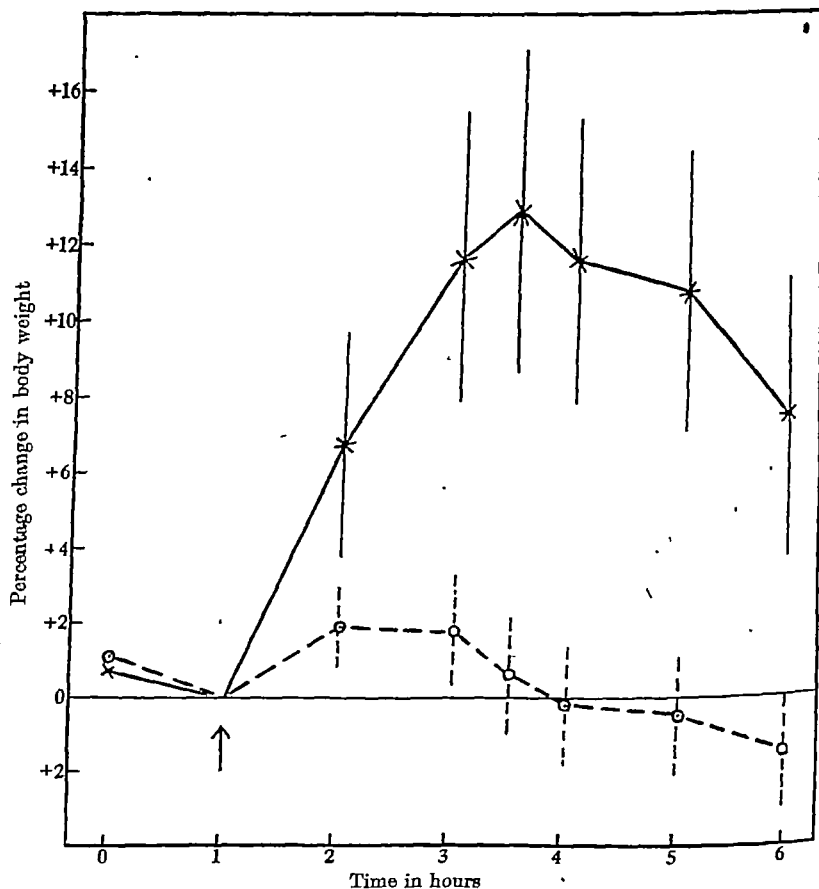


Fig. 4. The difference between the effect on the water balance of frogs of an extract of a single frog pituitary gland and of 40 mU. pitocin. Note that the oxytocic activity of a frog pituitary gland amounts to considerably less than the equivalent of 40 mU. pitocin (see Fig. 3). X—X mean percentage changes in weight of twenty frogs injected with extract of one frog pituitary gland each. O—O same frogs injected with 40 mU. pitocin each. Injections at the time marked by arrow. t (for maximum increases of weight) = 2.58, $P < 0.02$.

The discrepancy between the average amounts of the antidiuretic principle (about 3.5 mU.) and of the oxytocic principle (less than 40 mU.) contained in the pituitary gland of the frog is more apparent than real.

The extracts used for the determination of the oxytocic potency were crude and contained small amounts of impurities which had an oxytocic effect. A slight fall of blood pressure was regularly observed when the extract of a frog pituitary gland was injected into the vein of a spinal cat (Fig. 5). This finding indicates the presence of histamine-like impurities which enhance the oxytocic potency of crude frog pituitary extracts. Fig. 5 also shows that the pressor effect of the extract of a single frog pituitary gland is negligible. It is certainly much less than that caused

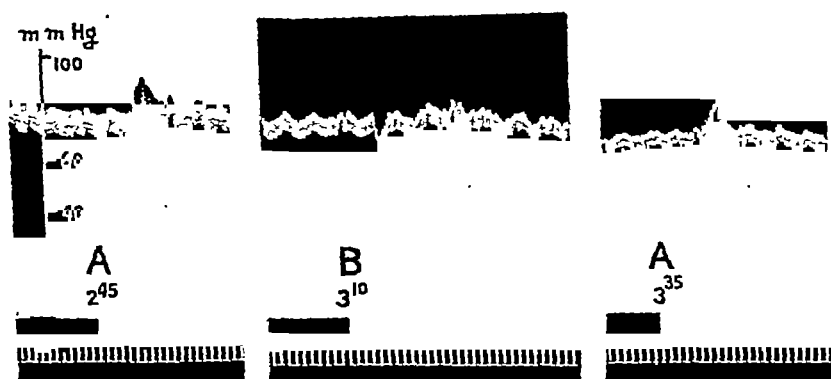


Fig. 5. Effect on blood pressure of spinal cat of extract of one frog pituitary gland. A=intravenous injection of 40 mU. pitressin. B=intravenous injection of extract of one frog pituitary gland. Time marker=10 sec. The frog pituitary extract contains less than the equivalent of 40 mU. of vasopressor principle. Note slight fall of blood pressure after injection of frog pituitary extract indicating presence of histamine-like impurity.

by 40 mU. of post-pituitary mammalian extract, i.e. a twentieth of the amount which has been shown (Fig. 2) to be just sufficient to reproduce the effect on the water balance of an extract of a single frog pituitary gland.

The content of water balance principle of the pituitary glands of various vertebrate classes

The assumption is made here that the relation of the activity of the water-balance principle to that of the oxytocic principle is the same in the B.D.H. extract used as in the international standard. Both were prepared from ox glands by a similar process. The unit of the water-balance principle is, therefore, defined as the amount of (frog) water-balance activity contained in 0.5 mg. of the international standard powder. It follows from this definition and from Fig. 2 that the pituitary gland of the frog contains approximately 800 mU. of the water-balance

principle. The antidiuretic activity of frog pituitary glands has been shown [Heller, 1941*a*] to be equivalent to approximately 3.5 mU. of pitressin. The ratio of water-balance activity to antidiuretic activity in frog glands is, therefore, approximately 228 to 1.0. The content of water-balance activity of the pituitary glands of representatives of the following classes of vertebrates was also determined. A. *Mammals*. The pituitary gland of rats was found to contain approximately 400 mU. of the water-balance principle (Fig. 6). The antidiuretic activity of a rat pituitary

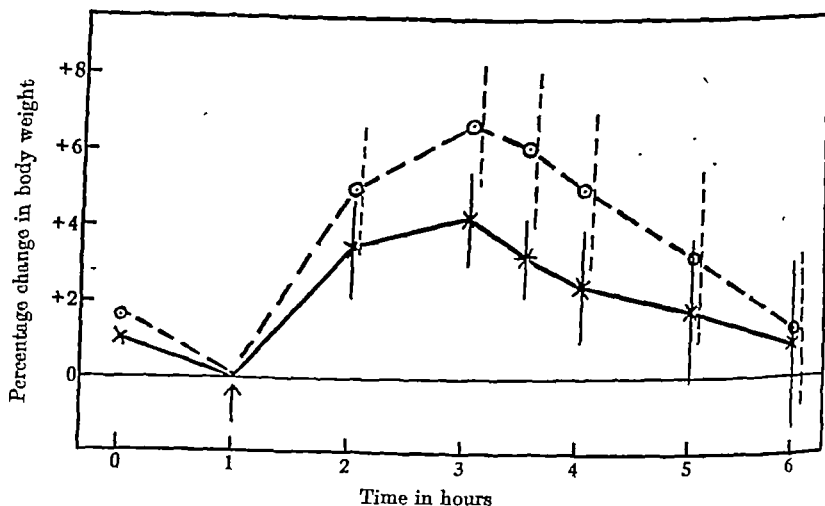


Fig. 6. Estimation of water balance activity of rat pituitary gland. X—X mean percentage changes in weight of ten frogs injected with extract of one rat pituitary gland each. O—O same frogs injected with 400 mU. of B.D.H. posterior pituitary extract each. Injections at the time marked by arrow. t (for maximum increases of weight) = 1.20, $P < 0.3$. It follows that rat pituitary glands contain an average of not more than 400 mU. of the water-balance principle.

gland has previously been shown to equal about 1075 mU. of pitressin [Heller, 1941*a*]. The ratio of water-balance activity to antidiuretic activity is thus 0.37 to 1.00. B. *Birds*. Pigeon pituitary glands contain about 1500 mU. of the water-balance principle (Fig. 7) and an average of 31.5 mU. of antidiuretic hormone per gland [Heller, 1941*a*]. The ratio of water-balance principle to antidiuretic principle is therefore 48 to 1.0. C. *Fishes*. It is known [Boyd & Dingwall, 1939] that extracts of fish pituitary glands are able to influence the water balance of frogs. However, quantitative determinations of the water-balance activity of fish pituitary glands are not extant. Fig. 8 gives an example of an experiment with cod pituitary extracts. It will be noted that the extract of one-tenth of a cod

pituitary gland had the same effect on the frogs' water uptake as the injection of 800 mU. of B.D.H. posterior pituitary extract. That is to say, the pituitary gland of the cod contained approximately 8000 mU. of the water-balance principle. The mean antidiuretic activity of a series of cod pituitary glands was shown to be equivalent to that of 166 mU. pitressin [Heller, 1941*a*]. The ratio of water-balance principle to antidiuretic principle is, therefore 48.0 to 1.0. This figure has to be accepted with the

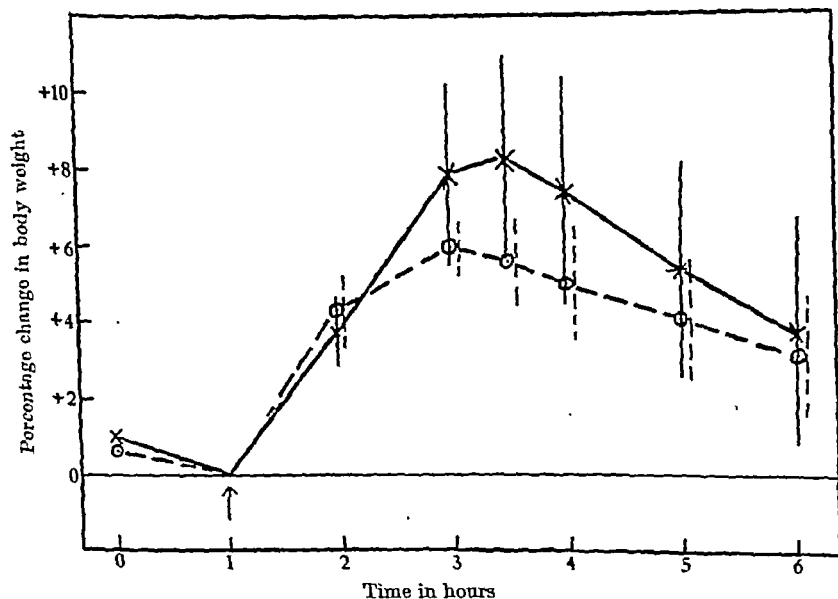


Fig. 7. Estimation of water-balance activity of pigeon pituitary glands. X—X mean percentage increases in weight of ten frogs injected with one-third of an extract of a pigeon pituitary gland each. O—O same frogs injected with 500 mU. of B.D.H. posterior pituitary extract each. Injections at the time marked by arrow. t (for maximum increases of weight) = 0.79, $P < 0.5$. It follows that pigeon pituitary glands contain about 1500 mU. of the water-balance principle.

reservation that the cod glands used for the water-balance assay were those of fishes which had not been freshly killed. The amounts of water-balance activity found are, therefore, likely to be too small. However, the figures obtained are sufficient to show that the ratio of water-balance activity to antidiuretic activity in fish pituitary gland is of a different order of magnitude from that of mammalian pituitary glands.

When comparing the water-balance activity of mammalian and non-mammalian pituitary extracts the objection could be raised that the lower water-balance activity of the mammalian extracts is apparent

diuretic potency of frog pituitary extracts to the level of mammalian pituitary extracts. An experiment on ten frogs gave the following figures: (a) maximum increase in body weight of frogs each injected with 50 mU. of 'specially prepared' pitressin = $1.4 \pm 1.73\%$. Weight at time of injection = 100%; (b) maximum increase in body weight of the same frogs each injected with the extract of one-third of a frog pituitary gland = $7.7 \pm 3.58\%$; (c) maximum increase in body weight of the same frogs each injected with the extract of one-third of a frog pituitary gland to which 50 mU. of pitressin had been added = $8.0 \pm 3.65\%$. The difference between (b) and (c) is not significant as $t = 0.06$ and $P > 0.9$.

The site of formation or storage of the water-balance principle in the pituitary gland

The experiments so far reported were performed with extracts of the whole pituitary gland. It was therefore impossible to say whether the water-balance principle was a posterior or an anterior pituitary hormone. The action on the water balance of frogs of mammalian posterior pituitary extract suggests the pars nervosa as the site of formation or storage in that class of vertebrates. In addition Biasotti showed in 1923 that mammalian anterior pituitary extract failed to influence the water balance of frogs. I repeated Biasotti's experiments using freshly prepared extracts of cat anterior pituitary lobes and obtained equally negative results. However, these findings do not necessarily apply to the pituitary glands of lower vertebrates. Frozen pigeon pituitary glands were therefore carefully divided into anterior and posterior lobe (the pars tuberalis and the tuber cinereum were included in the posterior lobe fraction), extracted in the usual way and injected into frogs. Fig. 9 shows an experiment on a series of twelve frogs. It will be noted that the anterior lobe extract had no discernible influence on the water balance. Essentially the same results were obtained with extract of the pars anterior of frog pituitary glands (Fig. 10). The pars anterior of frog glands was separated from the neuro-intermediate lobe in the following manner. The frog was decapitated and the skull opened from the dorsal surface. Using a dissection microscope, the olfactory lobes were pushed back with a blunt needle. The second and third nerves and the optic chiasma were cut and the brain pushed backwards until the pituitary gland became plainly visible. The anterior lobe was removed with the help of one sharp and one blunt needle, quickly rinsed in a drop of saline and put into a test tube containing 0.25% acetic acid. The remaining pituitary tissue was removed with a pair of fine pincers and the cranial cavity cleaned with a

small swab of cotton wool. The swab and the brain tissue proximal to the pituitary gland were added to the posterior lobe fraction. A comparison of Fig. 1 and Fig. 10 shows that extracts of the frog pars nervosa prepared

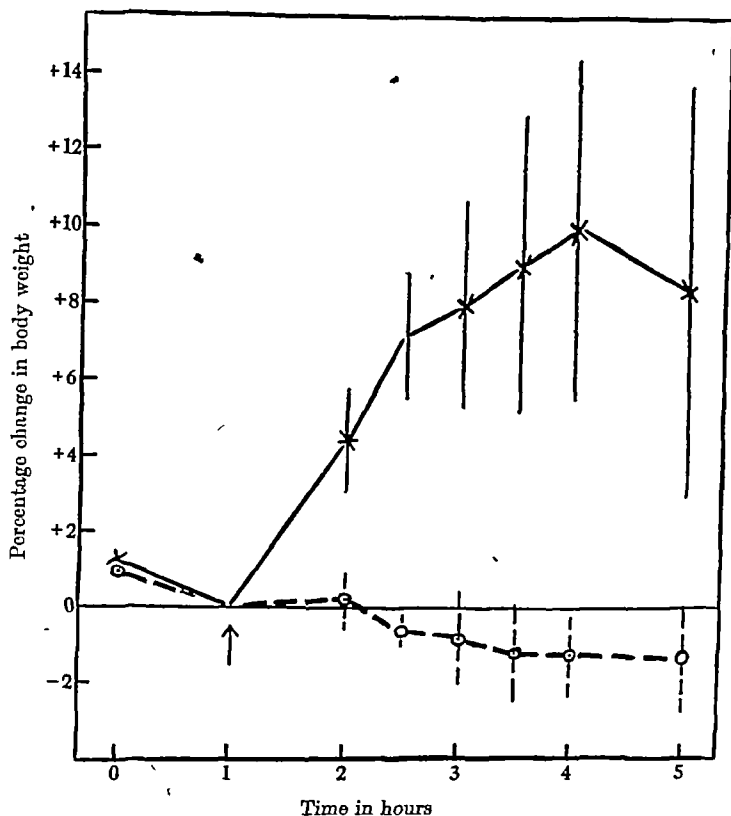


Fig. 9. The difference between the water-balance-activity of *pigeon* anterior and posterior pituitary extracts. X—X mean percentage changes in weight of twelve frogs injected with extract of one-third of a *pigeon posterior* pituitary lobe each. O - - O mean percentage changes in weight of twelve frogs injected with extract of a *pigeon anterior* pituitary lobe each. Injections at the time marked by arrow. t (for maximum changes of weight) = 1.97, $P < 0.1$. Note that the anterior lobe extract fails to increase the water uptake of frogs.

in this manner have quantitatively much the same effect as those of the whole pituitary gland, indicating that little of the active material had been lost in the process of separation. Fig. 10 shows also that extracts of the anterior lobe had no noticeable effect on the water uptake of frogs.

It seems sufficiently clear from these results that in the species of vertebrates investigated the posterior lobe must be regarded as the site of formation or storage of the water-balance principle.

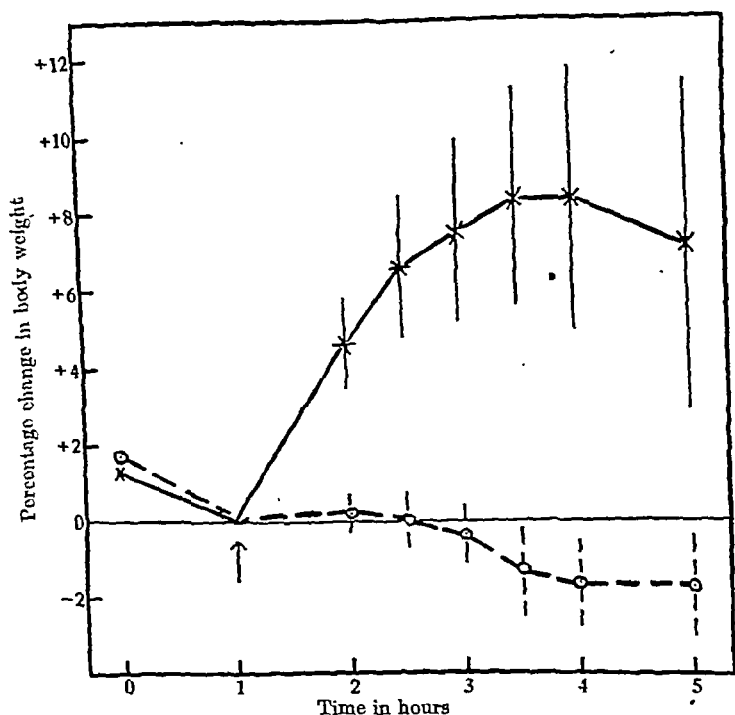


Fig. 10. Difference between the water-balance activity of frog anterior and posterior pituitary extracts. X—X mean percentage changes in weight of twenty frogs injected with extract of one frog posterior pituitary lobe each. O—O same frogs injected with extract of one anterior pituitary lobe each. Injections at the time marked by arrow. t (for maximum increase of weight) = 2.76, $P < 0.02$. Note that anterior lobe extract fails to increase the water uptake of frogs.

DISCUSSION

The results of the present enquiry show that the effect of pituitary extracts of lower vertebrates on the water balance of the frog is not proportional to their antidiuretic, vasopressor or oxytocic activity. Considering the magnitude of these disproportions it is difficult to see how the pituitary principle which causes an increase of the body weight of amphibians (water-balance principle) can be identical with either of the known posterior pituitary hormones. A chemical difference between the antidiuretic and the water-balance principle is indicated by the finding [Heller, 1930b and others] that the oxytocic and not the antidiuretic

vasopressor fraction of posterior pituitary extracts contains the bulk of the water-balance activity.

The water-balance principle was found in the pituitary glands of all vertebrates (mammals, birds, amphibians and fishes) investigated. A comparison of the effect of anterior and posterior lobe extracts of mammals, birds and amphibians showed clearly that practically all of the water-balance principle is formed or stored in the posterior lobe or its anatomical equivalent. The fact that bird anterior lobe extracts fail to influence the water balance shows further that the water-balance principle cannot be identical with the melanophore hormone. Birds have no intermediate pituitary lobe and De Lawder, Tarr & Geiling [1934] have shown that in this vertebrate class the melanophore hormone can be extracted from the anterior lobe. The results of the experiments with bird anterior lobe extracts are in agreement with Oldham's [1936] observation that highly purified (mammalian) melanophore hormone preparations fail to influence the water balance of frogs.

The quantitative distribution of the antidiuretic and of the water-balance activities in the glands of the various classes of vertebrates differs. Table 2 (last column) shows the approximate ratio of antidiuretic

TABLE 2. Comparison of 'water-balance' activity and antidiuretic activity of pituitary glands of representatives of various classes of vertebrates. The unit of the water-balance principle is defined as the amount of water-balance activity contained in one international unit of a mammalian post-pituitary extract. Figures for antidiuretic activities taken from an earlier paper [Heller, 1941a]

Species of vertebrate used	Milliunits of water-balance principle contained in one pituitary gland. (Figures in brackets = hormone content per 100 g. animal)	Milliunits of antidiuretic principle contained in one pituitary gland. (Figures in brackets = hormone content per 100 g. animal)	Ratio of water-balance principle to antidiuretic principle per gland
Rat	400 (238)	1075 (356.0)	0.37/1.00
Pigeon	1500 (429)	31 (7.2)	48.00/1.00
Frog	800 (4080)	3.5 (11.8)	228.00/1.00
Cod	8000 (—)	166 (—)	48.00/1.00

to water-balance principle in the pituitary glands of various classes of vertebrates. It will be seen that the pituitary glands of mammals contain very large amounts of an antidiuretic principle [Heller, 1941a] but relatively little of the amphibian water-balance activity. On the other hand, extracts of pituitary glands of lower vertebrates contain large amounts of the water-balance principle but have little antidiuretic activity. The position of mammals with regard to the two post-pituitary activities is thus in sharp contrast to that of any other class of vertebrates. Table 2

shows also that, surprisingly enough, as far as the hormone content of their posterior pituitary lobe is concerned, birds must be classed with the lower vertebrates.

The very different antidiuretic potency of mammalian and non-mammalian pituitary extracts does not appear to influence the assay for water-balance activity. Frog pituitary extracts to which amounts of mammalian antidiuretic hormone (pitressin) had been added which raised their antidiuretic potency to the level of a mammalian extract had much the same water-balance effect as normal frog pituitary extracts. On quoting figures for the ratio of antidiuretic to water-balance activity of pituitary extracts of different vertebrate classes the reservation must be made that antidiuretic activity was estimated by intravenous injection into rabbits whereas water-balance activity was determined by injection into a lymph sac of frogs. However, in view of the recent report of Ogden & Sapirstein [1940] that unspecific augmentor effects can be obtained equally with intravenous as with subcutaneous injections of posterior pituitary preparations, no attempt was made to estimate water-balance activity by another than the usual method.

It is impossible to decide at present whether the different proportions of antidiuretic and of water-balance activity found in the pituitary glands of the various classes of vertebrates indicate the distribution of a purely antidiuretic principle and of a factor which influences the 'water balance' only. The existence of an antidiuretic principle with a weak action on the water balance and of a water-balance principle with a weak antidiuretic action is a possible alternative. In other words there is no experimental evidence to prove that any of the posterior pituitary factors are secreted as separate chemical entities (Heller, 1941 *b*) though it may be possible to prepare them as such. The possibility must therefore be envisaged that the posterior pituitary glands of different classes (and even different species, see Geiling & Oldham, 1937) of vertebrates elaborate a secretion which exhibits the same activities but—by some unknown modification of the molecule—contains them in different proportions.

Our knowledge of the importance of the antidiuretic and of the water-balance activities for the water metabolism of the different classes of vertebrates is very fragmentary. The action of the antidiuretic hormone on the kidney of the mammal is clearly established, so is the action of the water-balance principle on the water metabolism of amphibians, but the effect on the water metabolism of either of the two principles on any of the other classes of vertebrates is either unknown or highly controversial. It will be noted (Table 2) that the amount of water-balance principle in

mammalian posterior pituitary lobes was smaller than that found in the glands of any lower vertebrate. However, this small amount may not be negligible and future research may reveal its physiological significance. On the other hand, it cannot be excluded that the presence of the water-balance factor in the mammalian gland is in the nature of a 'vestigial hormone' and that the post-pituitary regulation of the water metabolism in the mammal has been taken over by the antidiuretic hormone.

SUMMARY

1. Considerations are advanced for believing that the pituitary principle causing an increase of body water in the frog (water-balance principle) is not identical with any of the known posterior pituitary hormones. This assumption is based on the following findings:

A series of frogs injected with the extracts of one frog pituitary gland each showed a maximum gain of body weight of $12.4 \pm 3.94\%$ (S.E. of mean of seventy observations). The pituitary gland (including the pars tuberalis and the tuber cinereum) of the frog contains the following average amounts of the post-pituitary principles: an average of 3.5 mU. of antidiuretic activity, less than 40 mU. of vasopressor activity and less than 40 mU. of oxytocic activity. However, approximately 800 mU. of a mammalian posterior pituitary extract (containing the antidiuretic and vasopressor and oxytocic activities in equal proportions), had to be injected to produce an effect comparable to that caused by the injection of the extract of a single frog pituitary gland (Fig. 2).

2. The fact that the extract of a single frog pituitary gland contains sufficient of the water-balance principle to cause a marked change of the water balance of a frog suggests a physiological importance of this pituitary principle for the water metabolism of amphibians.

3. It would appear that the water-balance principle is a regular constituent of the pituitary gland of all classes of vertebrates.

4. A comparison of the effects of cat, pigeon and frog anterior and posterior lobe extracts shows that the water-balance principle is formed (or stored) in the posterior lobe of the pituitary gland.

5. The ratio of antidiuretic to water-balance activity contained in the pituitary gland differs in different groups of vertebrates. The results of the present inquiry suggest a unique position of the mammal, the pituitary gland of which contains large quantities of the antidiuretic factor but relatively little of the water-balance principle. The reverse is the case for the pituitary gland of lower vertebrates (birds, amphibians and fishes;

Table 2). A difference between the hormonal control of the water metabolism of mammals and of that of the other vertebrate classes is suggested.

I wish to thank Prof. J. H. Burn for providing facilities in his department, and Sir H. Dale and Prof. J. H. Gaddum for a valuable suggestion. I am much indebted to Dr Stanley White of the Parke, Davis Company for the supply of hormone preparations used in these experiments.

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TABLE 1. Effect of insulin in rats on normal diet

Group	Days on test	Units of protamine zinc insulin (total)	Calories of food daily, per rat	No. of rats	% wt. change	Insulin	
						Units/group of 10 rats	Units/1000 g. initial wt. of rats
I. Control: fed <i>ad lib.</i>	7	0	74	10	+6	28.2	11.2
Insulin injected: paired fed	7	7	74	10	+7	21.9	8.7
II. Control: fed <i>ad lib.</i>	6	0	77	7	+5	29.3	11.1
Insulin injected: paired fed	6	10	73	7	+1	7.9	3.0
III. Control: fed <i>ad lib.</i>	7	0	61	10	-3	24.6	8.5
Insulin injected: paired fed	7	13	61	7	-4	13.3	4.4
Insulin injected: paired fed	7	13	64	7	+0.4	9.3	3.1

1 unit on the last day, making a total of 10 units per rat. In the third experiment each rat was injected with 1 unit daily for the first 2 days, 2 units daily for the next 4 days and 3 units on the final day, making a total of 13 units per rat. When the larger doses were used some of the animals died. The results are shown in Table 1. They indicate that the daily administration of protamine zinc insulin causes a definite reduction in the insulin content of the pancreas. This fall occurs even though the animals are 'paired fed' with the control groups and show little or no decrease in body weight.

(2) The effect of insulin in starving rats

In this experiment the control and test animals were starved for 7 days. The rats of the test group received a daily subcutaneous injection of 1 unit of protamine zinc insulin. A considerable number of these animals failed to survive. The results of the determination of insulin content in those which survived are shown in Table 2.

TABLE 2. Effect of insulin in fasting rats

Group	Duration of fast days	Units of protamine zinc insulin per rat daily	No. of rats	% wt. loss	Insulin	
					Units/group of 10 rats	Units/1000 g. initial wt. of rats
Starved + insulin	7	1	7	26	4.2	1.4
Starved control	7	0	7	27	16.3	5.6
Starved + insulin	7	1	7	25	2.3	0.9
Starved control	7	0	7	29	8.9	3.5
Starved + insulin	7	1	7	23	2.1	0.7
Starved control	7	0	9	24	17.3	6.3
Average: Starved + insulin					2.9	1.0
Starved control					14.2	5.1

THE EFFECT OF INSULIN ADMINISTRATION ON
THE INSULIN CONTENT OF THE PANCREAS¹

BY C. H. BEST AND R. E. HAIST

*From the Departments of Physiology and Physiological
Hygiene, University of Toronto, Toronto, Canada**(Received 10 February 1941)*

It has been reported previously that fasting or fat feeding causes a definite reduction in the insulin content of the pancreas of rats [Haist, Ridout & Best, 1939; Best, Haist & Ridout, 1939]. It was thought that the administration of insulin to fasting animals or to those receiving fat might throw some light on the mechanism by which this effect was brought about. Several interesting facts have emerged. Insulin administration produces a decrease in the insulin content of the pancreas in fed rats and augments the fall in fasting or fat-fed animals.

METHODS

The procedures employed for the extraction of insulin from the pancreas and the determination of the potency of the insulin solutions were the same as reported previously [Best *et al.* 1939]. Male rats of the Wistar strain weighing between 200 and 300 g. were used and the pancreatic tissue from ten rats was taken for each test.

RESULTS

(1) *The effect of insulin in fed rats*

Several experiments were performed to demonstrate the effect of insulin administration in rats fed a balanced diet. The groups injected with insulin received the same diet and the same caloric intake as the controls. In the first experiment a daily subcutaneous injection of 1 unit of protamine zinc insulin was given to each rat. In the second experiment each rat received 1 unit of protamine zinc insulin daily for the first 2 days, 2 units daily for the following 2 days, 3 units on the next day and

¹ A preliminary account of part of this work appeared in *Science* (1940), 91, 410.

TABLE 1. Effect of insulin in rats on normal diet

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I. Control: fed <i>ad lib.</i>	7	0	74	10	+6	28.2	11.2
Insulin injected: paired fed	7	7	74	10	+7	21.9	8.7
II. Control: fed <i>ad lib.</i>	6	0	77	7	+5	29.3	11.1
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1 unit on the last day, making a total of 10 units per rat. In the third experiment each rat was injected with 1 unit daily for the first 2 days, 2 units daily for the next 4 days and 3 units on the final day, making a total of 13 units per rat. When the larger doses were used some of the animals died. The results are shown in Table 1. They indicate that the daily administration of protamine zinc insulin causes a definite reduction in the insulin content of the pancreas. This fall occurs even though the animals are 'paired fed' with the control groups and show little or no decrease in body weight.

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In this experiment the control and test animals were starved for 7 days. The rats of the test group received a daily subcutaneous injection of 1 unit of protamine zinc insulin. A considerable number of these animals failed to survive. The results of the determination of insulin content in those which survived are shown in Table 2.

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Starved + insulin	7	1	7	25	2.3	0.9
Starved control	7	0	7	29	8.9	3.5
Starved + insulin	7	1	7	23	2.1	0.7
Starved control	7	0	9	24	17.3	6.3
Average: Starved + insulin					2.9	1.0
Starved control					14.2	5.1

It will be evident from the table that the insulin content of the pancreas is reduced considerably below the fasting level when a daily injection of protamine zinc insulin is given to fasting rats.

(3) *The effect of insulin in fat fed rats*

In the first experiment in this group the control animals were fed fat *ad libitum* for 7 days and in the second experiment for 15 days. Actually, fat made up 90% of the diet, the remainder being composed of sugar and vitamins A, B₁ and D. The test groups received the same diet and the same caloric intake as the controls but in addition were given daily injections of 1 unit of protamine zinc insulin subcutaneously. The results are shown in Table 3.

TABLE 3. EFFECT OF INSULIN IN RATS FED ON FAT

Group	Days on test	Units of protamine zinc insulin per rat daily	No. of rats	% wt. change	Insulin	
					Units/group of 10 rats	Units/1000 g. initial wt. of rats
I. Control: fed fat <i>ad lib.</i>	7	0	10	-15	12.3	4.6
Insulin injected: paired fed with fat	7	1	10	-20	4.1	1.5
Insulin injected: paired fed with fat	7	1	9	-17	3.8	1.3
II. Control: balanced diet <i>ad lib.</i>	15	0	10	+22	22.2	9.6
Control: fat <i>ad lib.</i>	15	0	10	-15	8.5	3.7
Insulin injected: paired fed with fat	15	1	10	-14	2.0	0.8

These results demonstrate that the daily administration of protamine zinc insulin in fat fed rats leads to a much greater reduction in the insulin content of the pancreas than is obtained with fat feeding alone.

DISCUSSION

The average results of the experiments are illustrated in Fig. 1. It is evident that a fall in the insulin content of the pancreas is produced by fasting or the feeding of fat. In addition it is established (1) that the adequate daily administration of protamine zinc insulin causes a fall in the insulin content of the pancreas of rats receiving a balanced ration, (2) that the daily administration of protamine zinc insulin to fasting rats leads to a much greater reduction than is occasioned by fasting alone, and (3) that the daily injection of protamine zinc insulin in rats fed fat causes a much greater fall in the insulin content of pancreas than is

obtained in fat fed animals not receiving insulin. Histological studies on the tissues of these animals are now in progress.

The insulin content of the pancreas must be reduced by altering the relationship between the rate of insulin production and the rate of insulin liberation in pancreatic islet cells. In this instance insulin injections presumably reduce the need for insulin from the animal's own pancreas. It is improbable that the insulin liberation by the islet cells would be increased when insulin is given. The insulin production by the islet cells is presumably reduced. Since insulin administration enhances the effect

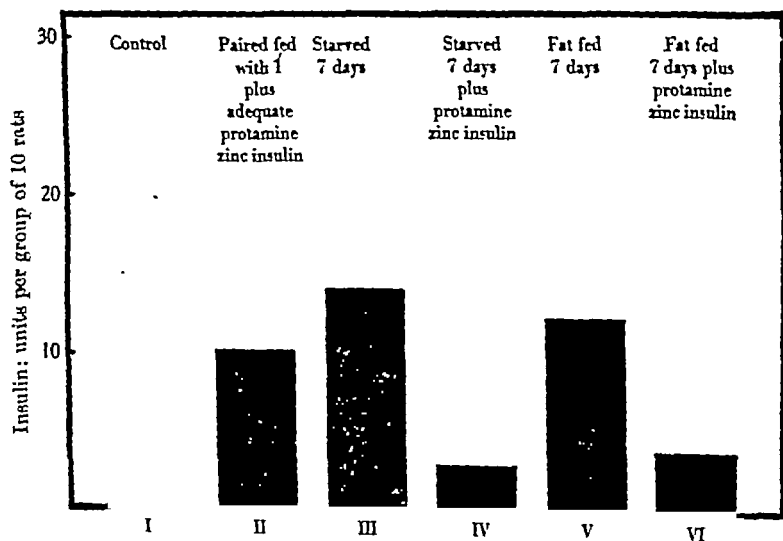


Fig. 1. Only experiments II and III of section (1) are included.

of fasting or fat feeding, the effects of insulin administration, fasting and fat feeding are similar. These procedures, i.e. insulin administration, fasting and fat feeding apparently rest the pancreatic islet cells by reducing the need for endogenous insulin. The results support Allen's conclusions regarding the effect of fasting which he derived from studies on partially depancreatized dogs [Allen, 1922] and are in accord with the results of Copp & Barclay [1923] and of Bowie [1926] concerning insulin administration in partially depancreatized animals. Our interpretation of these results is supported also by the recent report of Campbell, Haist, Ham & Best [1940] on the influence of insulin in preventing the changes in insulin content and the degeneration of islet cells which result from injections of diabetogenic anterior pituitary extracts.

SUMMARY

1. The insulin content of the pancreas of rats is reduced as a result of the daily injection of adequate amounts of protamine zinc insulin.
2. In fasting rats, the daily injection of protamine zinc insulin causes a much greater fall in the insulin content of the pancreas than fasting alone.
3. Daily injections of protamine zinc insulin in rats fed on fat lead to a much greater reduction in the insulin content of the pancreas than is produced by fat feeding alone.

We are indebted to the International Health Board of the Rockefeller Foundation for a grant which facilitated this work. The expert assistance of Miss Helen Bell is gratefully acknowledged.

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OBSERVATIONS ON THE RELATION OF HISTAMINE TO REACTIVE HYPERAEMIA

By H. KWIATKOWSKI

*From the Departments of Physiotherapy and Physiology,
Middlesex Hospital Medical School*

(Received 7 March 1940)

LEWIS [1927] made extensive studies on reactive hyperaemia and concluded that this phenomenon is caused by a vasodilator substance which accumulates in the tissue spaces of a limb when its circulation is obstructed. He suggested that the responsible agent ('H-substance') is a normal metabolite and is similar to histamine. Later, when methods for estimating histamine in blood became available [Barsoum & Gaddum, 1935*a*], evidence was obtained of an increase in the histamine equivalent in the blood of dogs, collected from the veins of a limb during a period of reactive hyperaemia [Barsoum & Gaddum, 1935*b*]. Other experiments showed that the increase in histamine equivalent occurs only in the plasma fraction of human blood [Barsoum & Smirk, 1936].

Reactive hyperaemia is a phenomenon of great physiological interest and is probably worthy of more extensive clinical use. The following experiments were performed to reinvestigate whether liberation of histamine is responsible for some of the symptoms of reactive hyperaemia. Histamine estimations on blood were performed before and during reactive hyperaemia in man and rabbits. The effect of cysteine on the fall of blood pressure due to reactive hyperaemia was studied, as this substance is known to antagonize the action of histamine on the guinea-pig's gut [Ackerman & Wasmuth, 1939].

METHODS

The method of Barsoum & Gaddum [1935] was employed for the extraction of histamine from human blood. In the case of rabbit's blood, which always contains a high concentration of histamine, the simplified modification of Code [1937] was used, in which the alcoholic

extraction is omitted. In all experiments bromthymol blue was used as indicator for adjusting the pH . The histamine was assayed on the guinea-pig's ileum:

Methods in man. Reactive hyperaemia was produced in normal male students. Blood was first collected from the cubital vein of one arm, which served as a control. The rubber cuff of a sphygmomanometer was placed round the other arm and inflated to above the systolic pressure to produce ischaemia for 10–30 min. After removal of the obstruction, 10 c.c. of blood were collected from the cubital vein of the arm during the first 1–3 min. of the reactive hyperaemia. In some experiments blood was prevented from leaving the limb during the period of reactive hyperaemia by means of venous occlusion at a pressure of 40–60 mm. Hg. In all experiments a marked red flush accompanied by a feeling of warmth was observed. All blood samples were collected in a 10 c.c. syringe and then immediately placed in trichloroacetic acid.

In other experiments the histamine in plasma and corpuscles was estimated before and during reactive hyperaemia. The blood was collected in paraffined syringes, transferred to paraffined centrifuge tubes and then immediately cooled in iced salt solution. After standing in the ice for exactly 5 min., the plasma was separated from the corpuscles, and, after being measured, was mixed with a 10% trichloroacetic solution and extracted in the usual way.

Preparation of rabbits. Animals of medium size were anaesthetized by an intravenous injection of nembutal into the ear vein. The abdominal aorta and inferior vena cava were isolated, and all arterial and venous branches, except the iliac arteries and veins, were ligatured. A cannula was introduced into a side branch of the inferior vena cava, from which 1 c.c. of blood was removed as a control. The arterial blood supply to the hindlimbs was interrupted for a period of 10–30 min. by obstruction of the lower part of the abdominal aorta and by means of a tight ligature round the base of the limbs.

Preparation of cats. Cats anaesthetized with ether and chloralose were eviscerated. All abdominal branches of the aorta and inferior vena cava, except those supplying the hindlegs, were ligatured. The mammary arteries were tied at the level of the 2nd–3rd rib. The injections of cysteine or histamine were made into the jugular vein.

Drugs. Cysteine hydrochloride and histamine acid phosphate were used. Doses of histamine are expressed in terms of histamine base.

Method of extracting histamine. Disagreement exists about the reliability of the method of Barsoum & Gaddum [1935]. Code [1937] was

able to recover by this means only 50–80% of the histamine added to water or blood. He found that the alcoholic extraction employed was responsible for the considerable loss of histamine. By his modified method, in which he omitted the alcoholic extraction, he was able to recover all the histamine added to water or blood.

A later publication, however, claims (contrary to Code) that 90–95% of the histamine added to Tyrode's solution or blood can be recovered by the extraction method of Barsoum & Gaddum [Anrep, Barsoum, Talaat & Wieninger, 1939]. Anrep *et al.* [1939] recommend the use of four successive alcohol extractions instead of three, as originally devised. When larger amounts of alcohol than 2 c.c. are used for each extraction, the yield is further improved.

In the present experiments only 75–80% of the histamine added to blood could be recovered though four successive alcohol extractions instead of three were employed.

New technique for assaying histamine. A new technique for assaying small amounts of histamine on the guinea-pig's ileum was successfully employed. This is based on the principle used for the detection of small amounts of 'sympathin' [Gaddum, Jang & Kwiatkowski, 1939]. A piece of guinea-pig's ileum was suspended in a small moist chamber which was maintained at a constant temperature by immersion in a suitable tank. A fine glass tube 12 cm. long passed through the warm outer tank; Tyrode's solution equilibrated with 5% CO₂ in O₂ flowing in a constant stream, dropped at regular intervals on the guinea-pig's ileum, the length of which was recorded isototonically with a magnification

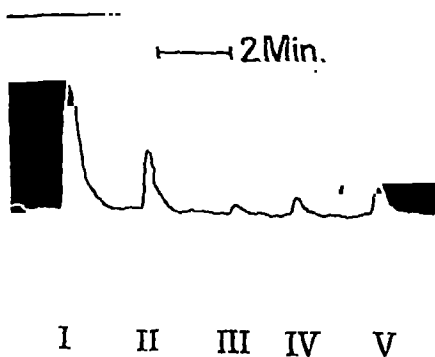


Fig. 1. Effect of various doses of 0.1 c.c. solution of histamine on guinea-pig's ileum perfused with Tyrode's solution. I, 0.01 μ g.; II, 0.005 μ g.; III, 0.001 μ g.; IV, 0.002 μ g.; V, 0.003 μ g.

of about 15. 0.1 c.c. of histamine or the solution of the extract was injected into the side tube of the small glass tube. Fig. 1 shows the effect of various doses of histamine. The preparation gave regular responses for a considerable time (1 hr. or more), provided that care was taken that the suspended gut was kept at a constant temperature, perfused at a constant rate, not twisted, and the lever well adjusted. Fig. 2 shows the influence of

the temperature and perfusion rate on the action of histamine. The best results were obtained at a temperature of 36°C . and a flow of 10 drops per 6 sec. The presence of bromthymol blue in the extract had no effect on the gut. Tyrode containing 0.01% CaCl_2 was used for assaying all blood extracts.

Fowl's rectal caecum. Two experiments were performed to try the effect of small doses of histamine on the perfused gut. The sensitivity was not found to be higher than that of the guinea-pig's gut.

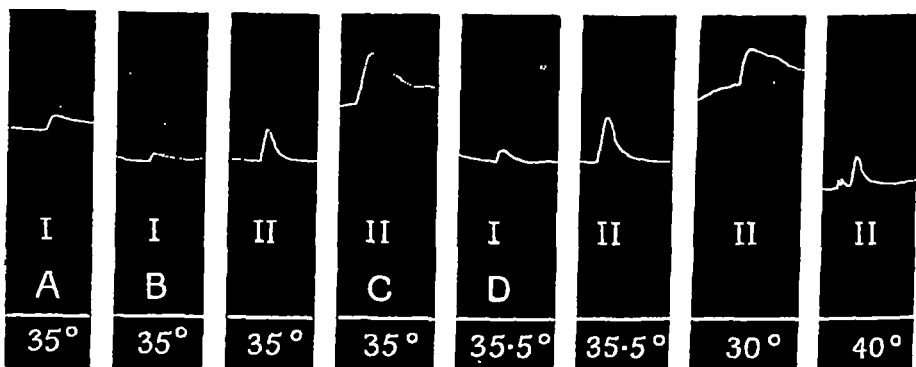


Fig. 2. Effect of temperature and perfusion rate on the response of guinea-pig's gut to histamine. The gut was perfused with Tyrode's solution. Doses of histamine solution in 0.1 c.c. I, 0.001 μg .; II, 0.003 μg . Rate of perfusion: A, 10 drops in 7.5 sec.; B, 10 drops in 4 sec.; C, 10 drops in 13.5 sec.; D, 10 drops in 6 sec.

Other organs. Small doses of acetylcholine were applied to the perfused leech muscle and frog's rectus abdominis. The leech muscle failed to relax after the contraction. The perfusion method was also not suitable for the estimation of acetylcholine on the frog's rectus abdominis.

RESULTS

Influence of calcium, glucose and atropine on the response of guinea-pig's gut to histamine

Calcium. Fig. 3 shows that normal Tyrode's solution is not the ideal fluid if maximal responses of the gut to small doses of histamine are desired. The response can be much enhanced if the CaCl_2 content is halved. The reduction of the CaCl_2 concentration in Tyrode's fluid produces an increase of tonus in the gut. In the complete absence of CaCl_2 there is no response to histamine, even if high concentrations of the drug are employed. An increase of CaCl_2 in the Tyrode's solution to two or three times the normal diminishes the response of guinea-pig's gut to histamine.

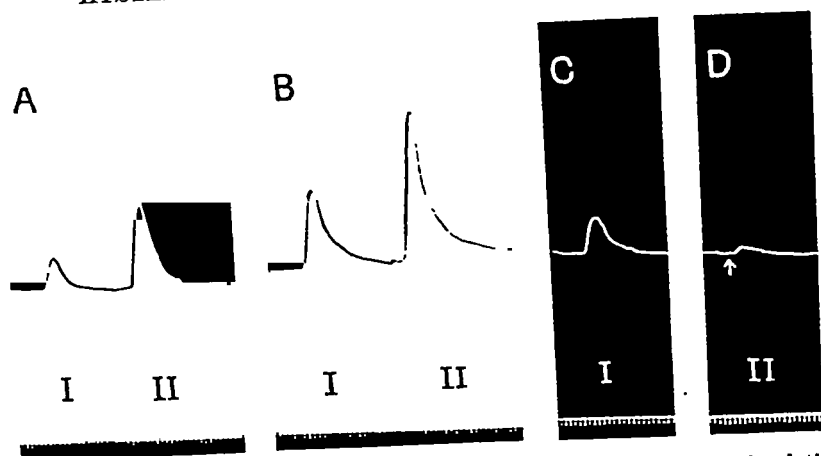


Fig. 3. Effect of histamine on guinea-pig's gut. A, perfusion with normal Tyrode's solution; B, perfusion with Tyrode containing half the normal CaCl_2 ; C, perfusion with normal Tyrode; D, perfusion with CaCl_2 -free Tyrode. 0.1 c.c. histamine: I, 0.005 μg .; II, 0.01 μg . Time in 10 sec.

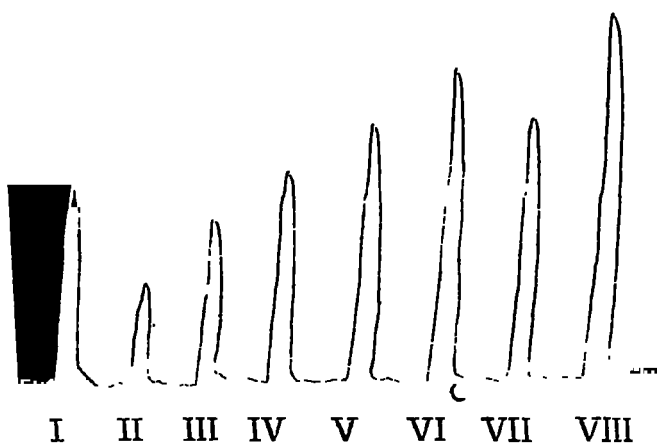


Fig. 4. Perfusion with Tyrode containing half the normal CaCl_2 . 0.1 c.c. histamine. I, 0.005 μg .; II, 0.002 μg .; III, 0.004 μg .; IV, 0.005 μg .; V, 0.006 μg .; VI, 0.007 μg .; VII, 0.006 μg .; VIII, 0.008 μg .

Fig. 4 shows the effects of various small doses of histamine on guinea-pig's ileum perfused with Tyrode's solution containing one-half the normal CaCl_2 content.

Glucose. Guinea-pig's intestine is very insensitive to histamine if perfused with glucose-free Tyrode's solution. The addition of glucose is followed by a marked increase of sensitivity to histamine.

Atropine. Addition of atropine to the perfusion fluid (1:10 million) diminishes the response to histamine. Atropine (10^{-4} – 10^{-5}) abolishes the action of histamine on the gut.

The nature of alcohol-insoluble smooth muscle contracting substance.

Anrep *et al.* [1939] found, in agreement with Code [1937], that the histamine equivalent of blood or of blood corpuscles extracted by Code's method is usually much higher than that obtained with the method of

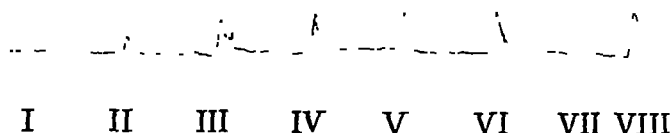


Fig. 5. Perfusion with normal Tyrode. I, 0.2 mg. KCl; II, 0.3 mg. KCl; III, 0.4 mg. KCl; IV, 0.1 c.c. extract derived from human blood corpuscles; V, The same after ashing; VI, 0.5 mg. KCl; VII, 0.1 c.c. extract derived from human blood plasma; VIII, 1 mg. KCl.

extraction devised by Barsoum & Gaddum. They state that this difference results from the presence in Code's extracts of an alcohol-insoluble substance which contracts the guinea pig's ileum and is included in Code's values for the histamine equivalent of the blood. This alcohol-insoluble substance is derived from the red blood corpuscles, and increases in amount on defibrination. It acts on the atropinized rectal caecum of the fowl (which has thus been rendered insensitive to acetylcholine) and is resistant to the action of histaminase. The presence of the excitator action on smooth muscle led Anrep *et al.* to suggest that the unidentified substance might possibly be identical with the adenylic compound described by Fleisch [1937]. This, however, cannot be the case, because, as Fleisch showed, this latter inhibits the guinea pig's uterus and does not stimulate it, as Anrep *et al.* stated.

In confirmation of Anrep *et al.* it was found that the alcohol insoluble substance can be detected in extracts derived from blood corpuscles, but not from plasma (Fig. 5). The unknown substance is still active on the guinea-pig's ileum after ashing. The inorganic nature of this substance and its presence in the blood cells suggested that the action might be due

to the presence of potassium which is known to contract smooth muscle [Mathison, 1911]. Table 1 shows the K content of the different extracts from corpuscles or plasma, as found by direct analysis. The same material, after ashing, was compared in respect of its action on the guinea-pig's

TABLE 1. Estimation of potassium in mg./c.c.*

	Extracts derived from	
	Alcohol-insoluble part	Alcohol-soluble part
I. Corpuscles	2.378	—
II. Corpuscles	2.273	—
III. Plasma	0.135	—
IV. Corpuscles	2.285	0.216

* We are very much indebted to Mr West of the Pharmaceutical Society of Great Britain who made the potassium estimations.

ileum with known solutions of KCl. As shown in Fig. 5, the K content so assayed was equivalent to a solution of KCl of 0.4–0.5 mg./0.1 c.c. or 2.0–2.5 mg. K/c.c. The agreement with the analytical data is therefore very close, and suggests that the action of the alcohol-insoluble substance is due entirely to its potassium content.

The concentration of histamine in human blood, collected from cubital veins under normal conditions, and during a state of reactive hyperaemia

Table 2 sets out the findings obtained. Histamine concentrations per c.c. are expressed in $\mu\text{g.}$ of whole venous human blood as estimated by the method of Barsoum & Gaddum. The amount of histamine, extracted from plasma and corpuscles of normal blood, and of blood during the early stages of reactive hyperaemia, is shown in Table 3.

TABLE 2. Histamine $\mu\text{g./c.c.}$

No.	Normal	Reactive hyperaemia
1	0.03	0.04
2	0.035	0.035
3	0.03, 0.03	0.03, 0.04
4	0.018, 0.02	0.025, 0.025
5	0.015	0.015
6	0.01, 0.01	0.01, 0.01
7	0.035, 0.045	0.035, 0.035
8	0.025, 0.025	0.025, 0.03
*9	0.07	0.075
10	0.025	0.025
11	0.03	0.035
*12	0.065	0.07

* Persons who suffered from hay fever.

Tables 2 and 3 show that no marked increase of histamine could be observed in any of the samples of blood withdrawn during reactive hyperaemia.

TABLE 3

	Normal concentration of histamine in $\mu\text{g./c.c.}$		Duration of circulatory arrest min.	Reactive hyperaemia; concentrated histamine in $\mu\text{g./c.c.}$	
	Plasma	Corpuscles		Plasma	Corpuscles
1	0.007	0.14	15	0.008	0.145
2	0.009	0.135	15	0.009	0.135
3	0.005	0.135	16	0.005	0.150
4	0.015	0.16	15	0.02	0.14
5	0.008	0.145	25	0.0085	0.135

Reactive hyperaemia in rabbits

Table 4 gives the values obtained for concentration of histamine obtained in experiments on rabbits. No increase of histamine could then be detected in the venous blood 1-7 min. after the restoration of the arterial blood flow to the limbs.

TABLE 4

Weight of rabbit (kg.)		2.6	2.5	3.25	4
Duration of ischaemia (min.)		20	20	20	30
Arterial blood	Time of collection of blood (min.)	Histamine $\mu\text{g./c.c.}$			
		3.0	2.8	3.5	1.5
Venous blood	Normal	3.2	—	3.5	1.5
	0-1	3.2	2.5	3.5	1.75
	1-3	3.2	2.5	3.5	1.7
	3-5	—	—	3.0	—
	5-7	3.2	2.5	3.0	1.5

Reactive hyperaemia and its effect on rabbits' blood pressure

Intravenous injection of histamine normally produces a rise of arterial blood pressure in rabbits, owing to constriction of the small arteries. We found that the restoration of the circulation in rabbits after 10 min. obstruction of the abdominal aorta is always followed by a pronounced fall of blood pressure.

Feldberg & Schilf [1930] obtained similar results and concluded that the H substance which is liberated in a rabbit after arterial obstruction is not identical with histamine.

The influence of cysteine on the effect of histamine and reactive hyperaemia

It has been shown that a number of amino-acids antagonize the action of histamine on the guinea-pig's gut [Ackermann & Wasmuth, 1939]. The action of cysteine has been attributed to substrate competition; the excess of free SH groups is supposed to block the tissue receptors, which otherwise would combine with the NH_2 group of histamine.

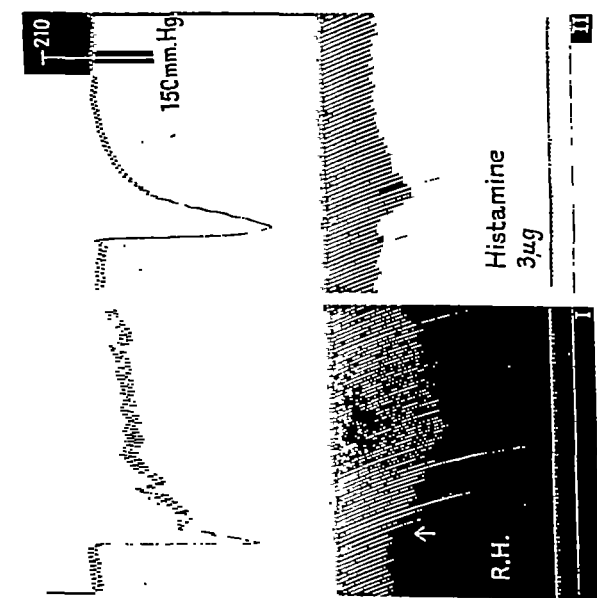


Fig. 6a.

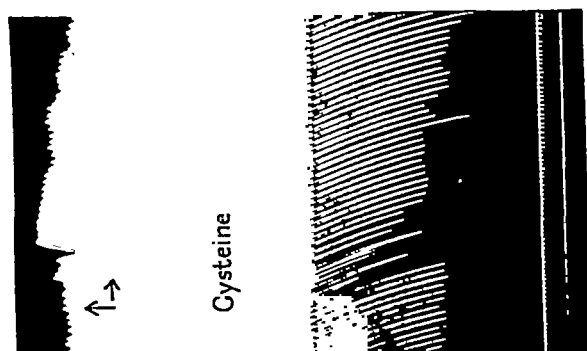


Fig. 6b.

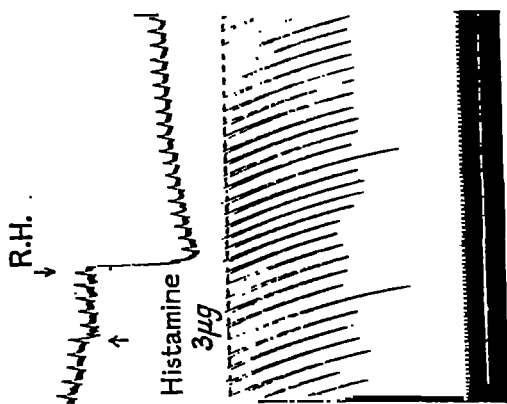


Fig. 6c.

Fig. 6. Cat (3.8 kg.) decerebrate. Records from above downwards are: carotid blood pressure, respiration, time in 10 sec. (a) I, lower abdominal aorta obstructed for 25 min. At arrow, release of obstruction, note fall in blood pressure. II, effect of injection of $3 \mu\text{g}$. of histamine. (b) At arrow, injection of 2.4 g . of cysteine hydrochloride dissolved in isotonic saline. (c) Lower abdominal aorta obstructed for 23 min. At first arrow inject $3 \mu\text{g}$. histamine intravenously; there is almost no change in blood pressure. At the second arrow (R.H.) release of obstruction; note fall in blood pressure.

Cysteine itself produces a contraction of the isolated guinea-pig's ileum. During this contraction, and very shortly afterwards, histamine does not act on the gut; but other drugs, such as acetylcholine or barium, are still effective. I confirmed these findings, and found that cysteine also antagonizes the action of histamine on cat's blood pressure. The amount of cysteine necessary to abolish the effect of histamine ($3-10\mu\text{g.}$) in cats is $0.6-0.8\text{ g./kg.}$ This dose was rather toxic in several experiments, and some cats died suddenly from acute failure of respiration and circulation shortly after the injection. The effect of a dose of $3\mu\text{g.}$ of histamine was compared in cats with the fall of blood pressure due to reactive hyperaemia before and after poisoning with cysteine. In the unpoisoned animal, although histamine produces a slightly greater fall of blood pressure, its effect on the depth of respiration is less marked (Fig. 6*a*). The administration of 2.4 g. of cysteine causes at first a slight rise of blood pressure (Fig. 6*b*), which is soon followed by a prolonged fall, accompanied by a depressor effect on respiration. The action of intravenously-injected histamine on the blood pressure is then annulled, but the fall of blood pressure after reactive hyperaemia still develops markedly (Fig. 6*c*). After 30 min. or one hour the effect of cysteine disappears, the blood pressure rises again, and the action of intravenously injected histamine is restored.

DISCUSSION

If the circulation to a limb is obstructed for a few minutes and the obstruction then removed, the skin shows within a few seconds a bright flush, increase of temperature and swelling. The remote effects consist of a fall of blood pressure and an increase in the depth of respiration. The local phenomena were attributed by Lewis to the action of the so-called 'H-substance' or histamine. In experiments on man and dogs, Barsoum & Gaddum and Barsoum & Smirk found an increase of histamine in blood withdrawn from a vein of the limb during a period of reactive hyperaemia.

Billings & Maegraith [1937] showed that histamine and adenosine accumulate in the venous blood returning from the ischaemic limb of rabbits and suggested that the accumulation of these substances, together with the acidity which develops, brings about dilatation of the small vessels of the limb, thereby establishing a good blood flow through the tissues within 12 hr. of tying the artery. Marcou, Comsa & Chiriceano. [1937] found only a negligible increase of histamine in venous blood during the first minute after release of an arterial obstruction of a limb in man.

In the present experiments on man and rabbits we were unable to find an increase of histamine in blood during reactive hyperaemia, although it was possible to estimate histamine doses as small as $0.001 \mu\text{g.}$ and less. To ensure greater accuracy, the collected blood in some experiments was divided and the histamine extracted in two different ways. One sample was extracted using Barsoum & Gaddum's method, and the other by the method devised by Code. Extracts obtained by Code's method include the potassium of blood. The extracts of venous blood during a state of reactive hyperaemia did not show higher values than the control samples. These results suggest that potassium too is not increased in venous blood during reactive hyperaemia.

Our results are not incompatible with the theory put forward by Lewis, who showed that the vasodilator substance responsible for reactive hyperaemia does not readily diffuse away from the site of action. This would be in agreement with our experiments on cats, where an injection of cysteine abolished the action of histamine on the blood pressure but had almost no influence on the fall of blood pressure caused by reactive hyperaemia. The fall of blood pressure after release of obstruction of the circulation to the hind legs of a rabbit cannot be due to the action of histamine, because this drug produces a rise of blood pressure. Our results suggest that the vasodilator substance formed, if it is histamine, does not diffuse away readily; the fall of blood pressure during reactive hyperaemia probably is due to the accumulation of blood in the dilated vessels of the limb.

Another possible explanation of reactive hyperaemia was discussed by Frey [1930]. He found that, during reactive hyperaemia, the pH of blood changes by $0.15\text{--}0.2$ to the acid side. The increase of pH in the tissues changes the kallikrein of blood from an inactive into an active compound. The latter produces the fall of blood pressure and increase of pulse amplitude. Frey showed that kallikrein previously injected into the ischaemic limb is changed into the active compound. These results, however, still await confirmation.

In conclusion, it may be said that, while a vasodilator substance is probably responsible for the local dilatation of capillaries and arterioles during reactive hyperaemia, histamine has not been shown to diffuse out in measurable amounts into the venous circulation. Our experiments do not support the view that the remote symptoms of reactive hyperaemia are due to the absorption into the circulation of histamine from the limb.

SUMMARY

1. A new technique is described for the assay of small amounts of histamine.

2. The nature of the alcohol-insoluble substance in blood which contracts smooth muscle was investigated, and was identified as potassium.

3. The histamine content of venous blood in man and the rabbit during a state of reactive hyperaemia was not significantly above the normal level.

4. The administration of cysteine annulled the action of injected histamine on the cat's blood pressure, but did not modify the fall of blood pressure which follows reactive hyperaemia.

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AFFERENT DISCHARGES TO THE CEREBRAL CORTEX FROM PERIPHERAL SENSE ORGANS

By E. D. ADRIAN

From the Physiological Laboratory, Cambridge

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IN a recent paper by Adrian & Moruzzi [1939] the electrical activity of the motor area of the brain was correlated with the discharge of impulses in the pyramidal tract. It was found that there was in general a close agreement between the larger potential waves of the cortex and the groups of impulses in the pyramidal discharge, and from this various conclusions could be drawn as to the activity of the cortical nerve cells. For further progress it seemed essential to have a survey of the sensory area made from the same point of view. A great deal is already known about the electrical activity of the various regions of the cortex which receive messages from the sense organs: the reactions of the visual area have been analysed in detail by Bartley & Bishop [1933], those of the auditory area in the cat by Bremer [1938] and of the sensory area in the monkey by Marshall, Woolsey & Bard [Bard, 1938], and general surveys have been made by Kornmüller [1937], by Gerard, Marshall & Saul [1936] and Derbyshire, Rempel, Forbes & Lambert [1936]. Much of the present work has merely confirmed what has been done already, but the recording of nerve impulses as well as cortical potentials can add some details to the picture of events taking place in the brain, and in existing circumstances it seemed advisable to publish what had been done. The greater part was written before the appearance of two important papers by Marshall [1941] and Marshall, Woolsey & Bard [1941] covering the same field. This must excuse the absence of detailed reference to the many points of agreement and to the few discrepancies between the present results and theirs. It is scarcely necessary to point out how much the present work is indebted to their earlier paper which showed the precise localization of electrical reactions in the sensory area.

METHOD

An essential feature of the method has been the use of a loud-speaker and amplifier system giving a faithful reproduction over a wide range of frequencies. The electrical changes in the cortex include both the very brief axon potentials due to impulses in nerve fibres and the much slower waves which are the characteristic product of the cortex. An optical system making a photographic record can be adapted to show one or the other but can rarely do justice to both simultaneously, whereas with a good loud-speaker it is easy to detect both impulses and waves.

Another essential in the study of cortical activity is the use of a variety of anaesthetics and of animals, for otherwise there is a grave danger of generalizing from results peculiar to one kind of brain or to one anaesthetic. The anaesthetics used have been dial and nembutal, urethane, chloralose, chloroform and ether, and ether alone and occasionally morphia. The animals have been rabbits, cats, dogs and monkeys, though the great majority of the experiments have been on cats. For the rest the technique has differed very little from that used for the motor area by Adrian & Moruzzi. One or more cortical electrodes have been held by a vulcanite support fixed to the skull. They have been either cotton-wool tufts for surface recording or else fine enamelled silver wires thrust into the cortex. An indifferent, earthed electrode is connected with the skin of the neck. Photographic records have been made with one or two Matthews oscillographs and a small cathode-ray oscillograph was used for visual observation at high speeds. Stimulation of the skin receptors was carried out by hand, a small camel-hair brush being used to move individual hairs. Pressure receptors were stimulated by weights placed on the foot. The period of stimulation was marked approximately by a hand-operated signal: the exact moment of contact was sometimes marked by arranging for it to produce an artefact in the oscillograph record.

RESULTS

Topography of somatic receiving area

In the following account the term somatic receiving area will be used to imply that area of the cortex which receives the afferent impulses relayed from tactile and other receptors of the body and limbs. The terms 'sensory area' or 'somaesthetic area' are often used in this sense, but as they sometimes bear a different meaning it seemed better to avoid them for the present.

By exploring the surface of the cortex with an electrode system leading to an amplifier and loud-speaker the regions which receive the afferent fibres can be mapped out with great ease. If the cortical electrode makes contact with any part of the receiving area a touch on the corresponding part of the body will give an outburst of impulses clearly audible as a brushing or hissing sound, and unless the activity of the cortical cells is greatly depressed there will be also one or more potential waves audible as a dull thud or rumbling. In light anaesthesia these may be obscured by continued trains of waves, and there may be repeated impulse discharges which make it difficult to be sure of the primary effect. It is best,

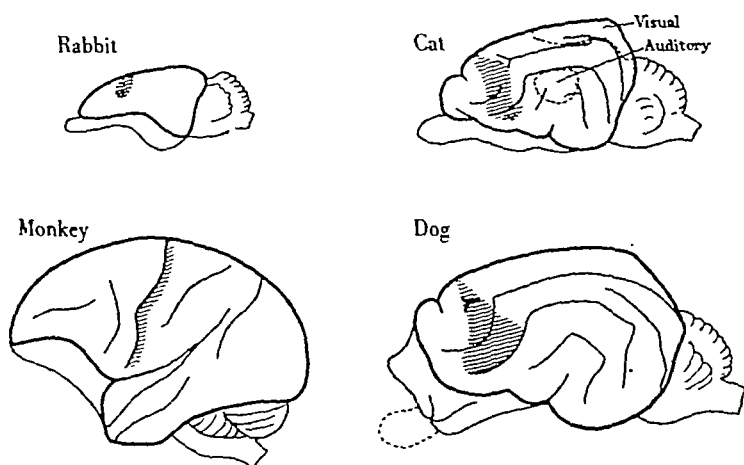


Fig. 1. Position and relative size of the area receiving afferent impulses from somatic receptors in the rabbit, cat, dog and monkey. The auditory and visual receiving areas are also shown for the cat's brain.

therefore, to restrict the degree of cortical activity by moderately deep anaesthesia, preferably with chloralose or one of the barbiturates, since these have little effect on the afferent pathways.

The position and relative size of the somatic receiving area in the rabbit, cat, dog and monkey are given in Fig. 1. The areas are those receiving impulses set up by mechanical stimulation, for touch, pressure and movement are the only stimuli which give a well-marked discharge to the cortex. It will be seen that there is a reasonable agreement with the areas determined from histological evidence, i.e. from the distribution of afferent projection fibres and from the cell structure of the cortex. Thus the areas for the cat and dog given in Fig. 1 coincide very closely with those shown by Campbell [1905]. The extent of the area is not affected by the depth of anaesthesia, except that there are marginal regions where

the discharge is only just audible under good conditions and becomes inaudible under bad. Again, there seems to be no essential difference between the area over which afferent impulses are heard and that giving the slower cortical wave response. The latter has been used by Marshall *et al.* to map out the area in the monkey, and the map given in Figs. 1 and 5 is in general agreement with theirs.

The cortical representation of different parts of the body is shown for the various animals in Figs. 2-5. The maps refer to particular animals though they were chosen to show the typical arrangement. The chief variation is in the extent to which the less important parts of the body (trunk, proximal part of limbs, etc.) are represented, but this does not affect the position of the main areas. In the rabbit (Fig. 2) it is often difficult to detect the arrival of afferent impulses in the cortex, for the potentials are never as large as in the cat, dog or monkey. Sometimes the

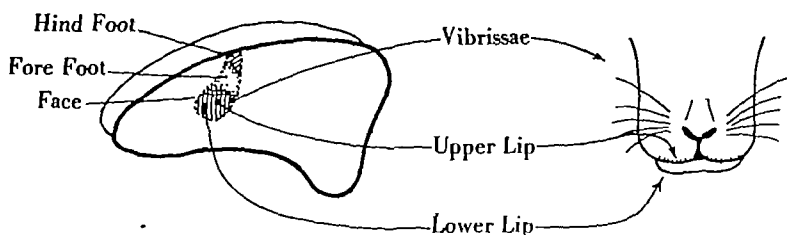


Fig. 2. Representation of different regions in the cortex of the rabbit.

only afferent effect which can be heard distinctly is the potential wave produced by touching the lips. In most animals impulse potentials and cortical waves can also be recorded from the vibrissae and usually from the fore- and hindfeet as well, but discharges from the limbs and trunk have never been identified. It will be seen that the localization follows the usual plan with the head ventral to the limbs, though the mouth-parts are represented in more detail than the rest of the body.

In the cat the most remarkable point is the double representation of the digits, impulses from the claws and ventral side of the toes appearing in a second area lying posterior to the face area and extending back into the ectosylvian gyrus (Fig. 3). The area for the claws of the hindfoot is small and easily overlooked; in a few animals it has never been found, but when present it is always caudal to that for the forefoot. No similar arrangement has been found in the dog or monkey, and it has been pointed out [Adrian, 1940] that its presence in the cat may be related to the special importance of the claws in the Felidae or may be concerned with the special mechanism for sheathing and unsheathing them. Certainly

stimulation of the area electrically or by strychnine produces well-marked flexion of the digits and protrusion of the claws provided that the motor cortex is intact and the anaesthesia not too deep.

Marshall *et al.* [1941] describe three distinct points in the cat's cortex which give a wave response when the dorsal surface of the forefoot is touched. Their 'point 1' lies within the anterior digit region shown in Fig. 3 and their 'point 3' within the posterior digit region, but their point 2 lies outside the digit regions as found in the present experiments and seems rather to fall within or near the region connected with the skin on the dorsal aspects of the wrist and forearm.

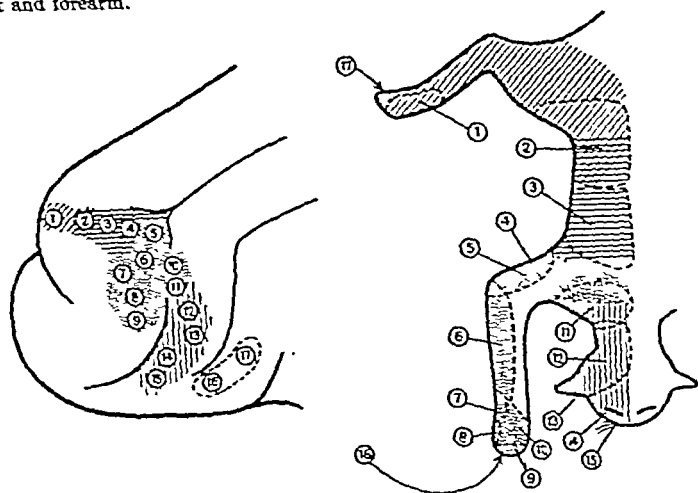


Fig. 3. Somatic receiving area in cat (exp. 10). The shaded regions show the fields for hindlimb, trunk, forelimb and head and the numbers indicate the particular areas sending impulses to different points on the cortex. The posterior digit and claw region (nos. 16, 17) is marked by a dotted line.

The discrepancy is more than a matter of anatomical detail, for Marshall *et al.* have previously found that some of the touch receptors on the monkey's hand are also represented, or give maximal potential waves, at three separate points on the cortex. Thus the existence of a triple representation in the cat would suggest some general principle of cortical arrangement. In the present experiments the double representation of the digits has seemed to be peculiar to the one species, and even there it has been very doubtful whether a single receptor sends impulses to more than one region in the cortex. The posterior region in the cat's cortex gives a response when the toe pads and the hairs round them are touched, particularly the pads on the thenar side of the foot. The anterior region responds to touching the hairs on the dorsal aspect of the digits, particularly those on the hypothelar side. Impulses reach both regions when the claws are tapped, but both toe pads, hairs and joints may then be stimulated. Elsewhere on the limbs and face a single group of hairs has not been found to send an afferent discharge to two separated points on the cat's cortex or to produce cortical waves at two points. In a few animals where the circulation in the cortex was poor there have been small inactive patches within the receiving area, so that a stimulus which was not sharply restricted would give potential waves on either side of the inactive region. But Marshall *et al.* have been at pains to confine

their stimuli to receptors in one region. The failure to confirm their finding may be due to the difference in the method of recording the cortical response, but it is difficult to see what particular factor can be responsible.

These results apply only to the cat's brain. In the dog and monkey no posterior digit region was found to correspond with that in the cat, but apart from this no detailed search was made for a double or triple representation in the cortex.

In the dog the arrangement resembles that in the cat except that the posterior claw area is absent and that the region giving impulses from the vibrissae and lips is particularly large. The localization of different parts within the face area is shown in Fig. 4. In both cat and dog certain parts of the body surface have never been found to send discharges to the exposed surface of the cortex, and with certain other parts the representation has varied from one animal to another. The mesial surface of the

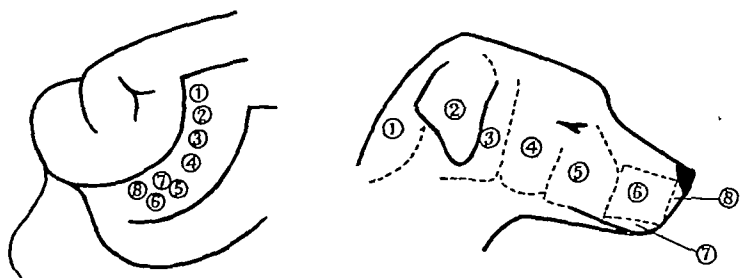


Fig. 4. Receiving area for the face in a dog. Numbers show cortical points connected with various parts of the face.

foreleg is in the former category and the trunk in the latter. It is, of course, unlikely that the mesial surface of the limbs would have much importance for the cerebrum, since they can have little contact with the outside world, but the buried area of the cortex in the coronal sulcus has not been thoroughly explored and it is possible that the missing parts are represented there though no discharges from them have been detected. It should be pointed out that the representation of the hindlimb and tail has not been worked out in any detail. Discharges from the anal region and the thigh were found on the mesial surface of the hemisphere in three cats and in the only dog in which the mesial surface was exposed.

Only two monkeys (*Rhesus*) have been investigated owing to difficulties of supply. Both gave results agreeing with Bard's map [Bard, 1938]. The receiving area did not extend forward on to the pre-central gyrus: it lay within the central fissure and over a strip about 3 mm. wide on the exposed surface of the post-central gyrus (Fig. 5). As in the cat and dog there was no evidence of bilateral representation even for the face. It is true that in light anaesthesia a sudden stimulus to

any part of the body may start a period of activity in the receiving areas of either hemisphere, but the characteristic afferent discharge is only obtained from the opposite side; and in deep anaesthesia an ipsilateral stimulus never gives any response, except on the trunk where the receptive skin field overlaps the mid-line by 1-2 cm. In all the animals there is some overlapping in the distribution of the afferent fibres leading from different parts of the body. In the monkey, for instance, from one spot on the cortex an afferent discharge may be obtained in response to

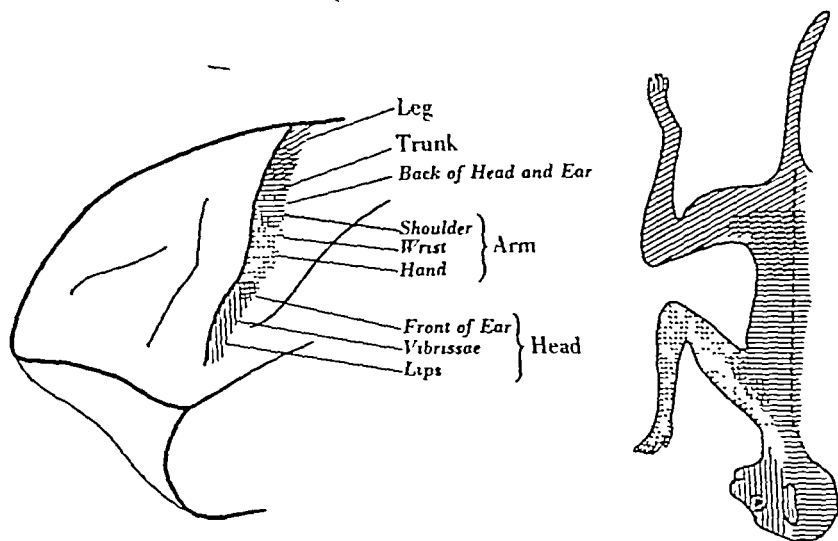


Fig. 5. Receiving area in a monkey (*Rhesus*). The trunk region includes the back of the neck and head [Marshall *et al.*].

tactile stimulation of points on a fairly large area of the abdomen and front of the thigh. And conversely, movement of a single hair will give a discharge audible over an area 1-2 mm. diameter on the cortex.

In the monkey the arrangement of the receiving area in a long, narrow strip emphasizes the serial representation of different parts of the body. But, as Bard points out, there is a marked departure from the segmental plan in that the cortical area for the back of the head adjoins that for the trunk, lying between it and the arm area. Since the back of the head is supplied by the upper cervical nerves we might expect that the points on the cortex connected with it would come between those for the face and those for the arm. It will be seen, however (Fig. 5), that the arm area intervenes between that for the face and that for the occiput, so that afferent impulses from the front and from the back of the ear arrive in the cortex at points separated by nearly 2 cm.

In the cat the segmental plan is followed more faithfully in that the neck and back of the head send impulses to the region adjoining the face area (Fig. 3), and the forelimb area intervenes between the area for the neck and that for the trunk. On the other hand, in the

cat and in the dog the area for the forefoot extends forward out of the regular line so that part of it adjoins the area for the vibrissae and snout. In the cat there is also the additional claw area which does not fit into any segmental plan.

The maps of the receiving area shown in Figs. 1-5, like those already published by Marshall *et al.* seem to represent a fixed anatomical arrangement of fibre tracts, for in animals of the same species there is very little variation in the general position and extent of the area, and in a given animal the area remains fixed in spite of great changes in blood supply, depth of anaesthesia, etc. It is not surprising that the arrangement of the afferent fibres should vary to some extent from one species to another and should not be strictly segmental, for the neopallium is not part of the primitive segmental nervous system and has developed differently in animals of different structure and habits. In each species the receiving area of the cortex is mainly concerned with those parts of the body surface which are most closely related to the outside world. In the rabbit the mouth-parts are the most important; in the cat the predatory claws have a region to themselves, and the dorsal and lateral aspects of the forelimb and trunk supply an area larger than that for the face; in the dog the face and mouth-parts are again more important though the limbs and trunk are better represented than in the rabbit.

The fixed distribution of fibres to the receiving area contrasts with the great variability in the distribution of activity in the cortex after the primary afferent discharge has reached it. A later section will deal with this activity, which may vary from a single localized wave to a prolonged rhythmic oscillation over the greater part of the hemisphere. Meanwhile the afferent discharge itself has to be discussed.

EFFECTS OF STIMULATION

Part 1. The afferent discharge to the cortex

With an electrode on the surface of the cortex the general character of the discharge can be made out although it is not possible to distinguish individual axon potentials. The movement of a few hairs will give an audible response, but the maximum effect is obtained by a light tap with the finger on the face or forefoot of the animal: a large number of tactile receptors are then stimulated simultaneously and the discharge is audible over a wide area. From many points on the surface of the body it is not possible to elicit more than a brief afferent volley. There are some regions, however, notably within the snout and forefoot areas, from which a more sustained impulse noise can always be produced by a continued (pressure) stimulus. With dial, urethane or chloralose the depth of anaesthesia

seems to make very little difference to this afferent discharge. With chloroform and ether there is more effect on the afferent pathways, and the discharge may fail entirely in moderately deep anaesthesia.

More detailed information is obtained by recording with a wire electrode from individual afferent units. An enamelled silver wire of gauge 44 or 46 (s.w.g.) was generally used, though a thicker wire sometimes gave good results. The wire is fixed to a rod sliding through a ball-and-socket joint so that it can be pushed gradually into the cortex; it is advanced until the sound of the impulse discharge breaks up into a succession of loud clicks, and further manipulation may then bring out a single series of brief potentials, alike in size and duration, and so presumably due to a single unit. In the cat such discharges are to be found at various depths from the surface, ranging from 1 to 5 mm. or more.

Axon potentials detected in this way are not all derived from afferent fibres, for if the electrode leads from the deeper layers of the grey matter some of them may be coming from cells in the cortex. But in an animal under dial it is easy to distinguish the afferent discharge, for the impulses which arise from the cortex bear a much less direct relation to sensory stimuli and often occur quite independently of stimulation. They are also much more dependent on the condition of the cortex, for they are abolished by cooling it, treating it with novocain or depriving it of blood by occluding the carotids, and they become grouped into high-frequency outbursts under the influence of strychnine. None of these procedures has much effect on the primary afferent discharge, and this is directly related to the sensory stimulation of a particular part of the body surface. In animals under chloralose or chloroform the distinction between afferent and efferent effects cannot be made out so clearly, but there is no indication that the afferent discharge differs in any important respect from that under dial.

The discharge due to pressure. The most straightforward results are those obtained by stimulating the pressure receptors, for the discharge lasts long enough to allow measurements of impulse frequency and regularity. The number of afferent fibres involved is also not as great as with tactile stimuli, and it is therefore easier to record from single units.

Typical records of the afferent discharge to the cortex are given in Figs. 6 and 7. In both the stimulus was pressure applied to the forefoot. Fig. 6 shows the gradual decline in the frequency with a constant stimulus and Fig. 7 shows the gradual increase when the pressure is slowly increased. In these records the general appearance of the discharge which reaches the cortex differs very little from that of the discharge

which leaves the receptor and travels up the afferent nerve fibre. It is true that the impulses reaching the cortex are not as regularly spaced as in the familiar stretch receptor records, and it is often difficult to obtain a wide range of frequencies to graded pressure. For this the character of

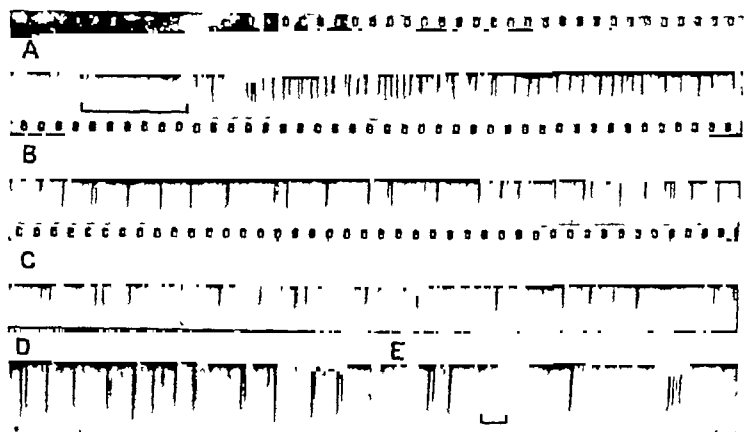


Fig. 6. Afferent impulses reaching the cortex as a result of pressure on the forefoot. Cat under dial. Single unit discharges recorded with wire electrode in the cortex. Record A shows onset of stimulation (white line above). B was made after 35 sec. stimulation and C after 60 sec. D and E are portions of A and C on a large scale to show the high frequency groups. Time shown by black line, in A 0.1 sec., in E 0.01 sec.

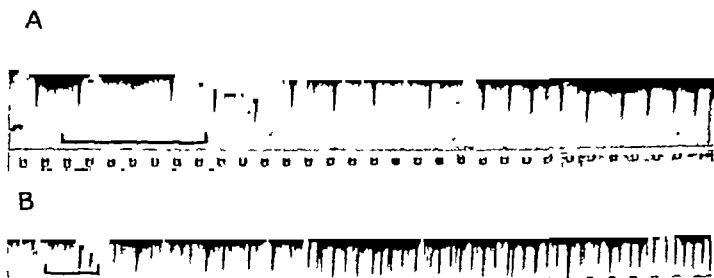


Fig. 7. Single unit discharge showing the effect of gradually increasing pressure. Records from two cats, both under dial, with wire electrode in cortex. The frequency rises to 60 a second in both. Black line gives 0.1 sec. in this and all following records.

the receptor may be partly responsible, for the impulses set up by pressure are seldom as regularly spaced as those from muscle spindles [Adrian & Zotterman, 1927], there is often a more rapid adaptation and it is more difficult to vary the frequency. In one respect, however, the discharge in the cortex may show an important difference: in Fig. 6 it will be seen

that many of the impulses come in groups of two or three very closely spaced with longer intervals between the groups. This kind of grouping is not always present—it is absent in the records in Fig. 7, but it has been found before in the fibres of the pyramidal tract [Adrian & Moruzzi, 1939] and seems to be a characteristic nerve cell reaction, each wave of activity lasting long enough to set up a repetitive discharge in the axon. The tendency for nerve cells to give high-frequency repetitive discharges is greatly increased by convulsive drugs, facilitation by electric stimuli, etc. In this case therefore it may depend on some abnormal state of the thalamic cells, and it is not certain that the kind of grouping shown in

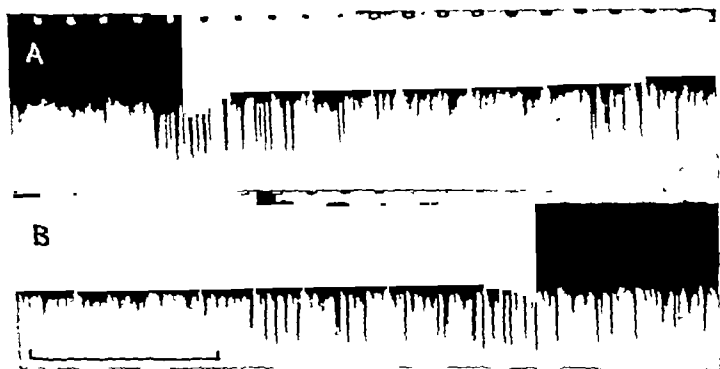


Fig. 8. Afferent impulses due to movement of vibrissae of snout. Cat under ether. In record A the hair is moved quickly up and down. In B it is moved down more slowly.

Fig. 6 would appear in the afferent discharge of a normal, unanaesthetized brain. All that can be said is that in animals under dial or chloralose brief multiple outbursts of this sort are common enough.

In records where such outbursts occur the frequency of the impulses can scarcely be compared with that in a discharge in which the impulses are evenly spaced, but apart from the outbursts the maximum frequency is considerably lower than in a peripheral nerve fibre, rarely exceeding 100 a second except for very short initial periods. With steady pressure the frequency has remained well above resting level for as long as 120 sec. The rate of decline varies greatly however. In some preparations prolonged search fails to detect any units in which the discharge persists for more than a few seconds. In others, in equally deep anaesthesia, there are many units giving a lengthy and well-graded discharge. The difference probably depends on the extent to which the thalamic part of the afferent pathway has been affected by the anaesthetic.

Pressure is the only form of stimulus which has given sustained discharges to the cortex. The receptors are to be found mainly in the pads of the forefoot and at the sides of the toe joints (a region where there are many Paccinian corpuscles). Stretching a muscle of the forelimb, though causing prolonged activity in the peripheral nerve, has never given more than a brief discharge to the cortex. Holding one of the large vibrissae of the face in a bent position has sometimes given a discharge lasting 5 sec., though movement of the ordinary hairs gives only a very brief effect. Fig. 8 B shows an afferent discharge in the face area produced by bending one of the vibrissae slowly; in Fig. 8 A a rapid up-and-down movement gives two brief outbursts at much higher frequency.

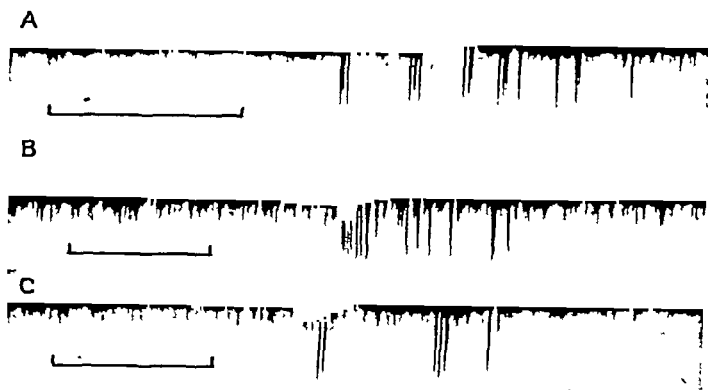


Fig. 9. Afferent impulses due to touch. A, cat under dial, touching dorsal surface of forefoot. B, cat under ether, touching hairs below eye. C, cat under chloralose, touching wrist.

The discharge due to touch. In the cat's cortex afferent discharges in response to thermal stimuli and to pain have not been detected: apart from the pressure receptors the tactile organs of the skin and hairs are the main source of the messages which reach the cortex, and discharges due to touch can be obtained from every part of the receiving area. When a wire electrode is used it is often difficult to be sure how many units are concerned, since the discharges are too brief to allow the sequence of impulses to be analysed, but in Fig. 9 A, B and C the larger spikes are all alike and so are probably derived from a single source.

As with the pressure response the tactile discharge to the cortex does not differ greatly from that in the peripheral nerve fibre, for in the latter the impulses are often spaced at intervals of only a few milliseconds and the whole discharge may be very brief. In most of the fibres to the cortex

it is very brief indeed (0.05 sec. or less) when the foot is tapped, and if it lasts longer there is a tendency for the impulses to appear in several high-frequency groups rather than in a continuous series. This may be seen in Fig. 9. The grouping recalls that found in the pressure discharge though it may be due merely to a similar grouping in the peripheral nerve fibre or to several discharges converging on to one thalamic neurone and reaching their maximum at slightly different times. The amount of convergence is considerable, for in the cat one afferent unit in the cortex may be activated by touching any group of hairs within an area 7 cm. square on the back. On the forefoot the convergence, in terms of skin area, is much less: in one unit, for instance, impulses could only be produced by touching the hairs covering an area 3 mm. square between the toes.

The afferent (thalamic) after-discharge. In the cat, dog and monkey if the anaesthetic is not too deep a discharge from the touch receptors to the cortex is often followed by a characteristic after-discharge. This consists of a succession of short volleys of impulses occurring at a frequency of between 10 and 20 a sec. and producing an oscillating noise in the loud-speaker. The after-discharge has been found with dial, chloroform and ether or morphia but not with chloralose and rarely with nembutal. The extent to which it is separated into distinct volleys varies considerably but seems to be greatest under moderately deep dial. It is most prolonged when the afferent pathway has been at rest for some time and is then made to transmit a considerable volley from the receptors. It is brief or entirely absent if the primary afferent discharge itself lasts more than a second or two. The duration of the after-discharge also varies with the depth of anaesthesia as well as with the magnitude of the afferent volley. The frequency of the outbursts changes very little, though the rhythm is slower in deep anaesthesia.

It is easier to study the after-discharge by listening to it than by making photographic records, for in these the impulse volleys become confused with the slower cortical waves and with impulses in cortical neurones. A wire electrode in the superficial layers of the grey matter (less than 2 mm. deep) will usually avoid the latter but will rarely show individual impulses of the after-discharge. The records in Fig. 10 give a good idea of the effect though the frequency is on the low side as the anaesthesia was deep.

The volleying character of the after-discharge shows that it is made up of synchronous or nearly synchronous outbursts from a collection of neurones which have been activated by the primary afferent volley. It is true that a single volley of impulses reaching the cortex may start a

period of rhythmic activity in the cells there, and the impulses coming from these cells would then be heard as an after-effect of the afferent volley. But there is no doubt that a rhythmic after-discharge can also arise in the afferent pathways before the cortex is reached, for in many preparations the repeated volleys can be detected in the exposed white matter and they remain audible in the cortex after the cells there have been put out of action by novocain or occlusion of the carotids. The effect does not develop below the thalamic level, for the sensory pathways can be tapped by a wire electrode at the level of the pons and there is then no trace of an after-discharge. It must therefore be due to thalamic neurones discharging more or less in unison as a sequel to the primary volley.

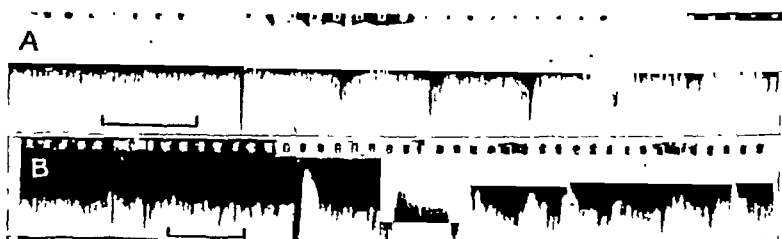


Fig. 10. Afferent (thalamic) after-discharge. Both records from cats under dial with wire electrode in the cortex. Stimulation by single touch to the forefoot. The primary afferent volley is followed by a series of volleys at intervals of 0.1 sec. or less.

The neurones concerned seem to be on or close to the direct pathway from the sense organs to the cortex, for the primary discharge and the after-discharge have the same distribution in the receiving area. The neurones must also be capable of reacting with one another, for otherwise the impulses would not be so well grouped. There is also some evidence that they may discharge spontaneously; for in light dial anaesthesia oscillating discharges are often heard from time to time in the receiving area, apparently in the afferent fibres and in the absence of deliberate stimulation. It is true that the general level of excitation in the thalamus may need a constant stream of impulses from the receptors to maintain it, but it is not at all unlikely that the thalamic cells which are normally set into rhythmical activity by an afferent volley should tend to discharge in the same way from time to time although no volley reaches them. They would then be displaying their affinity with the cells of the cortex which tends also to give rhythmic groups of waves under dial. The rhythm of the corresponding thalamic activity is somewhat faster

(15 a sec. as against 10 a sec.), but the character of the discharge certainly suggests that the same kind of mechanism is concerned in both cases.

In light anaesthesia in addition to rapidly oscillating discharges there is often a continued noise of impulses in the whole of the somatic receiving area. The noise is not as loud as that due to a tactile volley, but it seems to be caused by persistent discharge from the thalamus, for a wire electrode in the white matter will pick up continued trains of impulses at 10-25 a second. It is uncertain whether they correspond to particular afferent signals from the periphery, for it is usually impossible to find particular sense organs which will control them. They may represent a generalized excitation from the proprioceptors, since they can sometimes be modified temporarily by altering the position of the limb, although not by manipulation of particular muscles. The effect is present, though less distinct, in deep anaesthesia, for the noise derived from the receiving area still differs from that of neighbouring regions in having more high-frequency components.

Part 2. The effect of the afferent discharge on the cortex

The afferent cortical wave. When the foot or the face is touched, the afferent volley may influence the electrical activity of the cortex in a variety of ways, but almost invariably it will set up a brief potential wave in the somatic receiving area with its first phase surface positive. This initial wave has often been described and many of its properties are known. It occurs in the auditory area [Bremer, 1938; Bremer & Dow, 1939] and visual area [Bartley & Bishop, 1933] as well as in the somatic receiving area [Bartley & Heinbecker, 1938], and is produced by an electric stimulus to a nerve trunk [Forbes & Morison, 1939] as well as by a touch to a few hairs [Bard, 1938]. Examples of the initial wave in the different areas are given in Fig. 11. Prolonged stimulation by pressure or by rubbing the skin may give an initial wave if the afferent discharge has an abrupt onset, and during a sustained discharge there may be small, irregular oscillations which have some of the properties of the initial wave, but the analysis of these later events is more difficult and the characteristic response needs an abrupt stimulus to evoke it.

The most significant feature of the initial wave is that it is very closely related to the afferent discharge, so closely that it may be called the afferent cortical wave. As far as can be gathered by combining oscillograph and loud-speaker observations it has the same localized distribution within the receiving area as the afferent impulses, and

though it is reduced by failure of blood supply, deep chloroform anaesthesia, etc., it can nearly always be detected as long as the impulses are still audible. In light anaesthesia it may be hard to distinguish against a background of continued potential change, and the initial positive phase may be greatly cut down by the early development of a change in the opposite sense (surface negative). For this reason the wave is usually more obvious in deep anaesthesia with dial, nembutal or chloralose where the negative swing develops later or not at all. Sometimes there is a genuine increase in the positive phase as anaesthesia deepens, and there are considerable variations in the relative prominence of the afferent

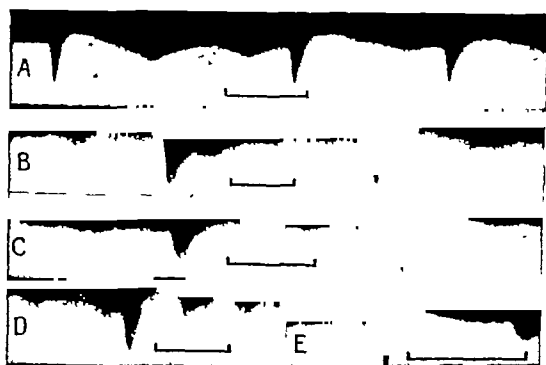


Fig. 11. Afferent waves recorded from the surface of the cortex. Downward movement indicates a positive potential at the cortical electrode in this and all subsequent records. A. Cat. Touches to forefoot. B. Rabbit. Touches to upper lip. C. Cat. Record from dorsal surface of medulla showing similar afferent wave in dorsal columns produced by touching foot. D. Cat. Record from visual area, showing similar wave produced by illuminating the eye. E. Cat. Auditory area. Afferent wave response to a click.

wave and afferent impulse noise in different animals. But in spite of this the wave appears as a relatively stable event depending mainly on the size of the afferent volley and not much on the state of the cortex.

In very deep anaesthesia the wave is monophasic or nearly so, with a potential (from a tap on the foot) of 0.1–0.5 mV. and a duration of 0.01–0.03 sec. In lighter anaesthesia with chloralose the positive phase is still large but it is followed by a lengthy negative phase. The whole wave complex then seems to be due to the brief afferent effect with a slower activity developing soon after it has begun and leading to a superimposed potential change which is mainly surface negative. This slower and later activity cannot be made to repeat itself at such short intervals as the afferent wave, so that if the touch is repeated 5–10 times a second

the negative phase disappears (Fig. 12). With dial in moderate doses a negative phase appears earlier, lasts for a shorter time and is less affected by rapid stimulation. Cutting down the blood supply to the cortex causes a much greater reduction of the later (negative) than of the initial (positive) activity, though the difference is not so great with dial as with chloralose. Convulsant drugs, on the other hand, cause a greater increase in the later activity than in the initial wave.

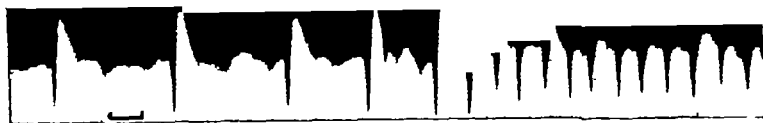


Fig. 12. Afferent waves in a cat under chloralose produced by repeated touches to forefoot, showing diphasic response changing to monophasic when the frequency of stimulation is increased to 10 a second. Downward movement (first phase of the response) indicates a positive potential at the cortical electrode.

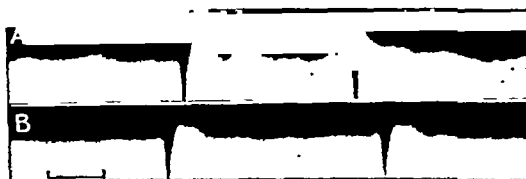


Fig. 13. Afferent waves recorded from the surface of the cortex (record A) and from the exposed white matter after removal of the cortex (record B). Cat under dial. Stimulation by touching foot.

The later activity will be considered more fully in the next section. Its much greater dependence on the state of the cortex suggests that it is due to neurones whose cell bodies are in the grey matter and are separated by one or more synapses from the afferent fibres. The appearance of an efferent impulse discharge in association with the negative wave supports this view (cf. p. 177). On the other hand, the initial surface positive wave seems to be due entirely to potential changes taking place in the afferent fibres. Its sign is compatible with an activity travelling from the deeper parts towards the surface of the cortex, but the best evidence of its origin was supplied by Forbes & Morison [1939] when they showed that a similar wave could be recorded from the underlying white matter by a needle electrode. As the waves which they described were evoked by electrical stimulation of a nerve trunk it seemed advisable to confirm the result with tactile stimulation. It is illustrated in Fig. 13 which gives the response to touching the foot in a cat deeply anaesthetized with dial.

The upper record is from the surface of the cortex. The whole of the grey matter of the motor area and somatic receiving area was then cut away: the exposed white matter was explored with the electrode until a point was found where the tactile discharge was again audible and the lower record was made from this point. It shows a potential wave scarcely distinguishable from that in the upper record, and as the lower record was made after destruction of the cortex we may reasonably conclude that the waves in the upper record were not the product of cortical neurones.

Evidence of this kind has led to a general agreement that the initial positive wave must be due to potential gradients in afferent fibres from the thalamus. The magnitude of the potential change at the surface of the cortex seems difficult to explain on the assumption that the wave represents no more than the summed action potentials in the afferent volley, but these might well be reinforced by changes of longer duration, e.g. after-potentials or depolarizations spreading from the nerve cells of the thalamus. The relatively constant time relations of the afferent wave are explained by the constant time relations of the afferent discharge from the receptors when the hairs are touched. This can be seen in a record from the medulla (Fig. 11 C). The potential wave there differs very little from that in the cortex except when the latter is cut short by an early negative swing. Both medullary and cortical waves can be given a slower rise and longer duration by manipulating the stimulus, but the range of variation is small, as the discharge must be abrupt if it is to give an adequate effect.

Cortical activity following the afferent wave. The events which succeed the afferent wave depend so much on the nature as well as on the depth of the anaesthetic that they are best described under separate headings for chloralose, dial and chloroform and ether. The main distinction is that chloralose slows down the activity of the cortical cells so that spontaneous waves occur at relatively long intervals, but it does not interfere with conduction from the afferent fibres provided that the cortical neurones have time to recover. With dial an afferent discharge may have much less effect, but in medium doses there may be considerable spontaneous activity in the cortex. Chloroform and ether are less selective and have more effect on the afferent pathways. In light dosage they seem to cause little slowing or interference with conduction, and their chief effect on the cortex is to promote a rapid synchronous activity of the nerve cells.

(1) *Chloralose. The efferent wave.* With chloralose and in the absence of deliberate stimulation the receiving area shows no more than two or

three potential waves every second. They are usually monophasic and surface negative with a maximum potential of as much as 2 mV. and a duration of 0.2–0.08 sec. (Fig. 14). The sign of the potential wave implies an activity developing near the surface of the cortex, or travelling away from the surface towards the interior. In agreement with this a wire electrode in the deeper layers of the cortex will often pick up an outburst of impulses corresponding to each of these waves, and in the motor area, where similar waves occur, each one is associated with a discharge in the fibres of the pyramidal tract [Adrian & Moruzzi, 1939]. Thus the waves mark recurring periods of activity involving the larger pyramidal cells.

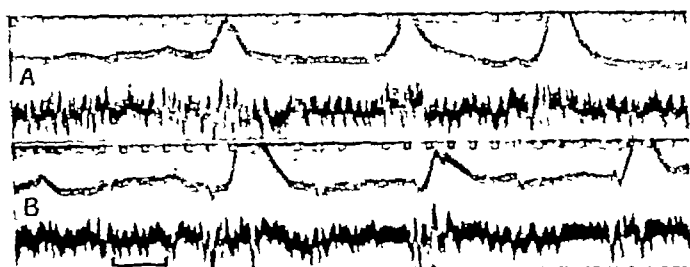


Fig. 14. Potential waves in chloralose anaesthesia occurring spontaneously (record A) or induced by tactile stimulation (record B). The upper tracing in both records is from the surface of the receiving area for the forefoot. The lower tracing is from a wire electrode in the pyramidal decussation. In A the three surface negative (effluent) waves are each accompanied by a pyramidal discharge. In B a pyramidal discharge occurs when the afferent (surface positive) wave is followed by an efferent wave.

With tactile stimulation repeated at short intervals the occasional negative waves are replaced by a succession of diphasic waves of the type already shown in Fig. 12. The first phase is then positive and coincides with the afferent volley to the cortex whilst the larger negative phase seems to be the equivalent of the spontaneous waves and like them is associated with an efferent discharge from the cortical cells (Fig. 14B). A cortical response of this sort may be regarded as a combination of the afferent wave with an 'efferent wave' which marks the successful excitation of the cortex and the discharge of impulses from the deeper cell layers. The efferent wave seems to have an initial positive component which sums with that of the afferent wave, but like the spontaneous activity it is mainly surface negative. The negative phase is certainly associated with the discharge of impulses by the cells, for if the stimuli are repeated at shorter and shorter intervals the negative phase and the efferent discharge are lost together and return together when the interval

is prolonged (Fig. 15). On the other hand, the negativity of the cortical surface greatly outlasts the discharge of impulses, so that much of the wave may be thought of as an after-potential. This may be seen when the potential wave in the motor area is compared with the discharge in the pyramidal fibres.



Fig. 15. Cat under chloralose. Upper tracing from surface of receiving area for forefoot, lower from wire electrode in pyramidal decussation. Four afferent waves (surface-positive) are evoked by touching the foot: the last two are at longer intervals and each gives rise to an efferent wave and a pyramidal discharge.

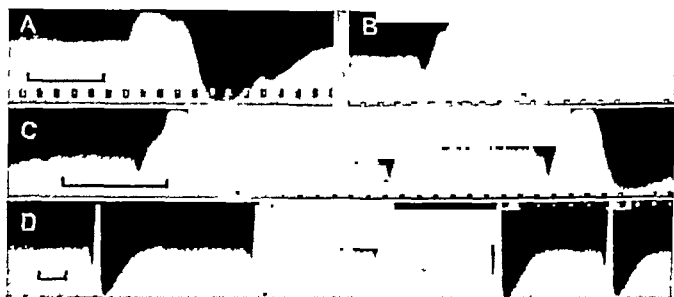


Fig. 16. Potential waves from the surface of the receiving area after local treatment with convulsant drugs. A. Cat under chloralose. Picrotoxin on cortex. Spontaneous wave with first phase surface-negative. B. Same animal. Compound wave evoked by touching the foot. First phase surface-positive. C. Cat under chloralose. Strychnine (0.5%) on cortex. Three touches to foot giving positive waves, the first and last followed by large negative waves. D. Cat under dial. Strychnine (0.5%) on cortex. Five touches to foot. The third gives only an afferent wave; the others give a complex response with a larger positive phase as well as the following negative phase.

Convulsant drugs. The same sequence of events is shown even more clearly after some part of the receiving area of the cortex has been treated locally with a convulsant drug like strychnine or picrotoxin. The anaesthetic in use is then immaterial, for with convulsant drugs the cortical neurones become readily accessible to the afferent discharge and give very large potentials, though they need a long time to recover between one excitation and the next. The efferent discharge can also be recognized easily when the effect is well established, for it is the characteristic high-frequency outburst described by Adrian & Moruzzi.

In the absence of intentional stimulation the area treated with the drug shows occasional waves which are initially surface negative (Fig. 16 A), as with chloralose. The chief difference is that the waves are much larger (up to 5 mV.) and are usually diphasic and the intervals between them are longer (2-10 sec.) with a steadier base-line. At each wave an outburst of impulses at a very high frequency can be detected in the deeper layers of the cortex and in the white matter. The discharge begins as the cortical potential starts to change but ends some time before the wave is over. With tactile stimulation there is again a close parallel with the response in chloralose, for if the touches are spaced 1 sec. or more apart each will produce the compound afferent-efferent response, beginning with a small surface-positive phase which is followed by a large negative one (Fig. 16 B). With a wire electrode it is often possible to hear both the initial afferent discharge and the high-frequency outburst from the cortex which follows it. With frequent stimulation or restricted blood supply the afferent discharge and the afferent wave can be made to appear without the efferent effect (Fig. 16 C).

It will be noticed that in the records in Fig. 16 D the initial positive phase of the response is larger when the efferent effect occurs than when the wave represents the afferent process alone. Apparently when the convulsive response is started by an afferent volley (though not when it occurs spontaneously) it begins with a surface-positive change which adds itself to that of the afferent wave. It is sometimes possible to detect the beginning of this second positive component, but more often all that can be seen is that the initial wave is much larger when the full response occurs. A similar increase produced by strychnine in the positive as well as in the later negative components has been found also with electrical stimulation of the optic nerve [Bartley, O'Leary & Bishop, 1937] and of somatic afferent nerves and of the fibres of the corpus callosum [Curtis, 1940]

(2) *Dial.* Under dial (or nembutal) there is a general tendency for the potential waves of the cortex to occur in groups with frequencies between 6 and 12 a second [cf. Bremer, 1935, 1936 and Derbyshire *et al.* 1936]. With light or medium dosage there is usually a continuous succession of waves over most of the cortex at a frequency which remains steady for considerable periods at about 10 a second or falls every few seconds to about half this rate. In deeper anaesthesia the slower waves drop out leaving the more rapid groups separated by periods of inactivity, or rather by periods in which the only sign of activity is a very small rapid oscillation.

With anaesthesia of medium depth whenever the large waves are not confluent it can be seen that the great majority of them make the cortical electrode negative to the indifferent, and when the waves are recorded in the motor area it is found that each is associated with a discharge of

as one series of oscillations and the groups of cortical waves as another series quite independent of the first. In some records it is even possible to distinguish the two as a series of positive and of negative waves, though they are often too close together for their sign to be recognizable.

The difference between the action of dial and chloralose is no doubt largely a matter of the range over which the afferent volley can effect the neurones of the cortex. In Fig. 17 (dial) neurones which are so situated in relation to the electrodes that they can influence the potential record are not affected by the afferent volley. In Fig. 12 (chloralose) all these neurones are affected by it. Theoretically some of them might be a long way from the

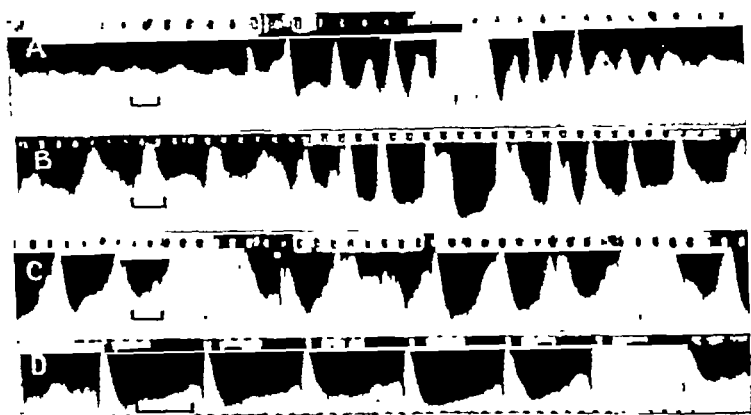


Fig. 18. Modification of cortical rhythms by afferent discharges in cats under dial. All records from the forefoot area. A. A single touch produces a train of waves at 10 a second. B. Pressure on the foot increases the size and frequency of the waves. C. A touch disturbs the rhythm momentarily (note diphasic afferent wave, marked by white dot above). D. Repeated touches impose their own rhythm on the cortical waves.

cortical electrode, for with monopolar recording a large group of neurones anywhere in the brain might produce a potential change. Actually the neurones responsible for the waves in Figs. 12 and 17 are not likely to be more than a few millimetres from the cortical electrode, for a local application of novocain will abolish the waves and local strychnine will replace them by waves of the large convulsive type. In fact the structures responsible for both the surface positive and the surface negative waves are probably very close together.

A dissociation at the level of the cortex was recognized by Bremer [1938] as a characteristic effect of dial and other anaesthetics of the barbituric series. In deep anaesthesia when there are long inactive periods between the groups of waves the dissociation is much less evident. The afferent volley has no longer to compete with the pacemaker which determines the spontaneous cortical waves and touches at suitable intervals will always start a period of rhythmic activity. It should be pointed out, however, that in deep anaesthesia the waves which follow

impulses in the pyramidal tract [Adrian & Moruzzi, 1939]. Thus the groups of negative waves under dial resemble the occasional waves under chloralose and indicate an activity spreading inwards from the surface and giving an efferent discharge. But there is the difference that under chloralose the electrical activity of a given point in the receiving area can be entirely controlled by stimulation of the appropriate receptors, for with a series of touches the occasional waves are replaced by a series of the complex afferent-efferent responses. With dial, however, the afferent volley rarely produces such a comprehensive effect. It is true that the initial wave is usually diphasic, with the first, positive phase cut short by a brief negative phase (cf. Fig. 17 B), and as the latter is absent in deep anaesthesia it probably indicates an excitation spreading to certain elements in the cortex. But this excitation, if it occurs, is often quite

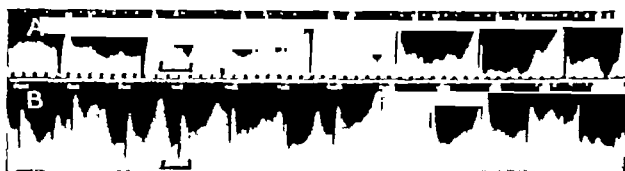


Fig. 17. Waves from the receiving area in cats under dial during repeated tactile stimulation. The brief afferent waves (initially positive) are superimposed on the slower negative waves of the spontaneous rhythm.

unable to influence the rest of the cortical mechanism in its neighbourhood, for the usual rhythmic waves may continue unchanged during the stimulation as though the afferent volleys had never arrived. The diphasic wave due to each volley is then superimposed on the slower and larger rhythm (cf. Fig. 17).

The dissociation of the cortex from the afferent pathways is not always so complete and in fact all degrees may be found, a common result of the volley being a tendency to a local increase in cortical activity rather than a specific and immediate excitation. Thus a tap on the foot may start a group of waves or may interrupt the rhythm, continued pressure may raise the frequency for as long as it is applied, and repeated tactile volleys usually succeed after a time in imposing their rhythm on the cortex (Fig. 18). These facilitating effects are more prominent when the cortex itself is less active; but there is very seldom the complete control of cortical activity in the receiving area which is found with chloralose or with convulsant drugs. Indeed in many preparations the tactile volley and the subsequent volleys of the thalamic after-discharge can be heard

were regular waves during stimulation but not in the period before or after.

When there are regular waves during stimulation their frequency seems to run more or less parallel with that of the impulses in the afferent units. But there is clearly a pooling of excitation from many fibres and an interaction of the excited neurones rather than a direct transmission from afferent fibre to cortical nerve cell. In the absence of stimulation, for instance, the cortex often shows a rapid rhythm (25 a second) superimposed on a slow (5 a second) or recurring at regular intervals, but no corresponding rhythm has been found in the resting afferent discharge. Again, during continued stimulation there is a far greater synchronization in the cortical than in the afferent neurones, for

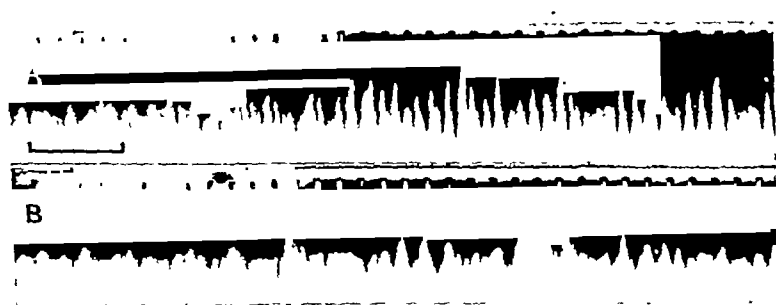


Fig. 23. Potential waves evoked by pressure to A, contralateral, and B, ipsilateral forefoot in a cat under light chloroform and ether.

very regular waves are evoked by a discharge which is heard as a non-rhythmic noise, and if a touch produces a grouped after-discharge from the thalamus the frequency of the volleys in it may differ considerably from that of the cortical waves which occur at the same time.

Excitation in other regions. Under light anaesthesia with chloroform or ether a touch, besides causing the localized excitation of a particular part of the receiving area, may produce a widespread increase of activity in other parts of the cortex. Thus the frequency of the waves in the fore-foot area of the right hemisphere may be increased by touching the right foot as well as the left, though the increase is not so great (Fig. 23). Similarly, the movement of one of the vibrissae of the face may increase the frequency in the foot area of either hemisphere. These secondary effects are most clearly seen when the cortex, though lightly anaesthetized, has passed into a period of relative inactivity: from this it can be roused, not only in the somatic receiving area but in regions farther back, and not only by tactile stimuli but by visual and auditory as well. In fact it

quency can sometimes be made to rise gradually from the resting to the stimulated value (e.g. from 10 to 60 a second); in fact the cortical waves can be made to vary over the same frequency range as the impulses in the afferent fibres.

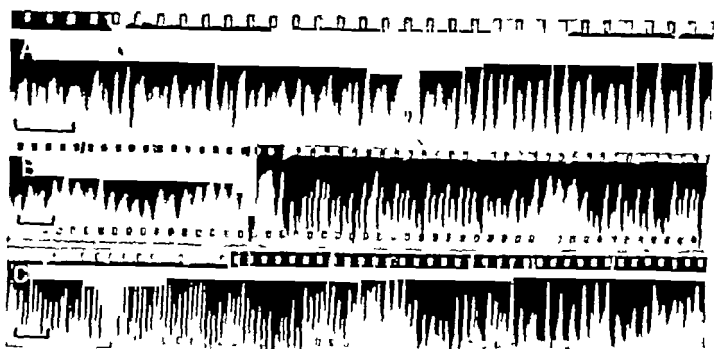


Fig. 21. Records from the receiving area in cats under chloroform and ether, showing the increase in frequency of the waves during stimulation. A. Gradually increasing pressure to forefoot. B. Another animal, pressure suddenly applied causes waves at 60 a second. C. Same animal as B. End of a pressure stimulation showing fall in frequency from 60 to 25 a second.

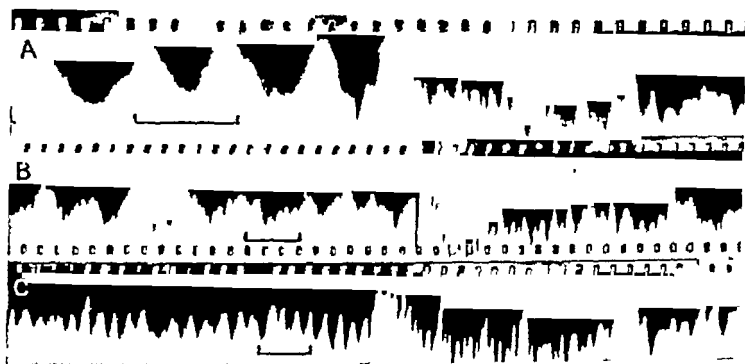


Fig. 22. Potential waves under chloroform and ether showing breakdown of regular rhythms by an afferent discharge. Records from three cats from the forefoot area of the cortex. Stimulation by pressure to the forefoot. Resting rhythm at about 10 a second in A and B, 40 a second in C.

Records illustrating the rise and fall in frequency on stimulation are given in Figs. 21 and 22. Some of them show also what happens when the synchronization is not maintained. In Fig. 22 A, for instance, there are regular waves at 10 a second before, but only an irregular and much smaller oscillation during the period of stimulation. In Fig. 21 A there

were regular waves during stimulation but not in the period before or after.

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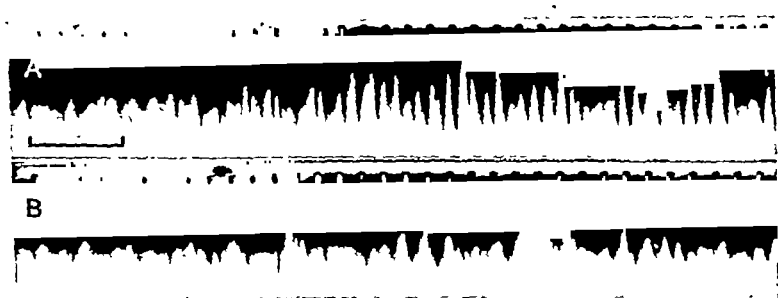


Fig. 23. Potential waves evoked by pressure to A, contralateral, and B, ipsilateral forefoot in a cat under light chloroform and ether.

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may become necessary to blindfold the eyes and avoid any noise when the effects of a touch are to be investigated.

In light anaesthesia a touch, sound or change of illumination may raise the level of activity in the cortex generally, but it is only in the appropriate receiving area, somatic, auditory or visual, that the noise of an afferent volley can be heard. Elsewhere there is sometimes an increase in the fainter rushing sound which forms a background to the thud of the cortical waves, but in parts of the supra-sylvian and marginal gyri there is rarely anything to suggest an increased afferent discharge. It does not follow that the increased cortical activity is not then due to impulses from the thalamus, for there is no reason to think that all impulses from the thalamus would be equally audible at the surface of the cortex. But the excitation of remote areas clearly depends on a mechanism which differs considerably from that responsible for the localized afferent discharges to the various receiving areas. The nature of this mechanism is beyond the scope of the present paper. There is evidently a clue to it in the work of Forbes & Morison on the widespread secondary cortical response which follows electrical stimulation of a nerve trunk, and the various possibilities are discussed in their papers.

DISCUSSION

As regards the afferent pathway the most striking result which has appeared in this work has been the fidelity with which the message from the receptors is handed on to the cerebral cortex. This applies only to the receptors for touch, pressure and hair movement, but when these are stimulated the noise of the discharge which reaches the cortex is not obviously different from what it would be in the peripheral nerves. With more exact recording certain differences are found; the impulses reaching the cortex may come in groups of two or three very close together and there may be a more rapid decline in frequency than there is likely to be in the discharge from the sense organ. With ether and chloroform the pathway may become completely blocked, but dial or chloralose seldom produce complete failure in spite of the synaptic regions which the signals must pass.

The phenomenon of masking found by Marshall *et al.* [1941] shows that there is a convergence of pathways on the way up to the cortex, and this has been confirmed. When a wire electrode leads from a single afferent unit it is often found that receptors over a considerable area can produce a discharge in it; and it is probable though not certain that a restricted stimulus may activate a number of afferent units. Thus the

connexion between the tactile receptors and the cortex is like that between the rods and cones of the retina and the fibres of the optic nerve, securing much convergence but allowing a spread of excitation in synaptic areas instead of limiting the signals to fixed and insulated pathways.

Such an arrangement might be expected to display inhibitory as well as excitatory effects, but so far no clear evidence of inhibition has appeared. The anaesthetic may be responsible for this, for the inhibition which Barron & Matthews [1935] have found in the spinal pathways was greatly reduced by anaesthetics. In light anaesthesia, however, provided that the pathway is unoccupied and has had time to recover, a touch on the face or the forelimb is signalled to the corresponding region of the cortex and the signal is not greatly changed en route.

But the primary signal is usually accompanied or followed by additional signals which it has aroused in its passage through the thalamus, and these are subject to much more variation. They are shown by the widespread activity which appears if the anaesthesia is light and the stimulus intense, and by the rhythmic after-discharge which follows the afferent volley. The mechanism of the widespread effect has not been investigated: that of the rhythmic after-discharge is made somewhat clearer by the proof that it can occur after removal of the cortex. Evidently it does not necessarily depend on a circulation of impulses from thalamus to cortex and back, and the thalamus must have its own mechanism for producing the after-discharge whether the cortex is there or not. The mechanism need not involve any special nerve-cell organization, for the same kind of reaction is found in other collections of excitable cells of quite different origin. In two papers by Eccles & Magladery [1937] on smooth muscle there are many records from the nictitating membrane showing a rhythmical after-effect essentially similar to that of the thalamus. The time scale is, of course, different, but as in the thalamus a single stimulus sets up an immediate response and this is followed by a series of waves due to the synchronous activity of many units and repeated at intervals which tend to settle down to a standard value. Evidently the smooth muscle fibres and the neurones of the thalamus have common properties which give them the same tendency to discharge in a synchronized rhythm.

The tendency can be seen in the cortical nerve cells as well as in those of the thalamus, for a single electrical stimulus to the cortex or an afferent volley (without an after-discharge) may set up repeated cortical waves. But the cortical neurones are more unstable than the thalamic and may continue their rhythmic activity for long periods. In the

thalamic neurones on the afferent pathway there are occasional rhythmic discharges in the absence of a deliberate stimulus and sometimes with chloroform or ether the neurones are in continued activity. But as a rule under dial an afferent volley is needed to set them off and their discharge seldom lasts for more than a second, whereas the neurones of the cortex maintain their rhythm for hours on end.

The thalamic after-discharge is sometimes non-rhythmic, and the primary discharge during continued stimulation by pressure or rubbing the skin seldom shows any dominant rhythm. Thus the thalamic neurones are associated in a way which makes for synchronous activity only when there are no local inequalities to prevent it, for local inequalities are more likely to be present during the actual transmission of a message than in the period of raised excitability which follows. The cortical neurones show

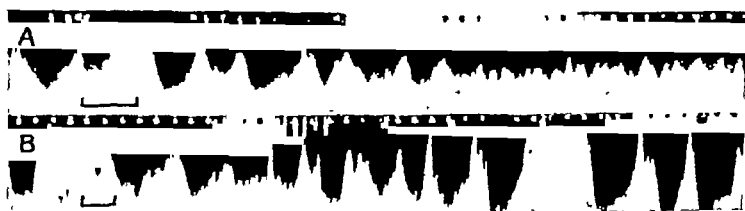


Fig. 24. A. Abolition of large waves by an afferent discharge in a cat under light dial anaesthesia. B. Increase in waves produced by an afferent discharge under deeper dial anaesthesia.

an association of the same kind. It is well illustrated by the records in Fig. 24 made from two cats, one lightly, the other deeply anaesthetized with dial. The potential waves are recorded from the forefoot area of the cortex and midway through each record a steady pressure is applied to the foot. In the lightly anaesthetized brain the synchronous beat is broken up into rapid, irregular wavelets, for in the light anaesthesia the impulses in the afferent discharge can exercise a more direct effect on the different neurones of the cortex. Deeper anaesthesia prevents this direct transmission but a general facilitation can still occur. The result is that the waves increase in size and frequency and their synchronization is not disturbed (Fig. 24 B).

The way in which the anaesthetized brain will react to an afferent discharge will vary according to the relative degree to which the anaesthetic has affected cortex or thalamus, excitability or rate of recovery, direct transmission or indirect facilitation, etc. In different animals there are considerable variations although every care may have been taken to

give the anaesthetic in the same way. In the unanaesthetized brain this particular difficulty would be avoided, but the afferent signals will arrive in an area in which the conditions vary from moment to moment owing to the continued rhythmic activity and vary over longer periods owing to the changing frequency of the waves. We need to know much more of the origin of these continued rhythms and of the factors which determine the general level of activity in the unanaesthetized brain. But the present study shows how profoundly the reactions of the brain are modified when spontaneous changes are reduced or absent. Under chloralose, for instance, the cortex is reduced to the level of a simple reflex mechanism. Spontaneous waves occur at a very low frequency but direct synaptic transmission is still possible, with the result that a signal arriving by the afferent pathway is passed at once to the motor area and down the pyramidal tract to the spinal motor neurones. Thus a tap on the foot causes a twitch of the leg by a reflex arc which includes the cerebrum. At each relay station the signal is reconstituted by fresh neurones but the alterations which occur in the cortex are of the same order as those on the way in or out.

Cerebral reflex arcs of the same sort can be produced by treating the surface of the cortex locally with strychnine, though in this case the signal is amplified on the way. The cortex can give such an immediate and direct response because it is doing so little on its own account. As soon as the effect of the drug and the anaesthetic passes off the grey matter will regain its normal instability and tendency to continued discharge, the motor neurones will be exposed to a changing pattern of excitations and inhibitions and direct reflexes will cease.

CONCLUSIONS

The area of the cerebral cortex which receives afferent signals from the somatic sense organs has been surveyed by recording the impulse discharges in afferent fibres as well as the potential waves in the grey matter. To avoid confusion the area is spoken of as the somatic receiving area. A variety of anaesthetics has been used.

1. The area receiving the primary afferent discharges from the thalamus agrees with the 'sensory area' determined histologically and with the area determined by recording cortical potential waves in deep anaesthesia. The representation of different parts of the body surface shows the same stability as that found by Marshall *et al.* using the potential wave method.

2. The receiving area of the cortex is concerned with those parts of the body surface which are most closely related to the outside world. In the rabbit the mouth parts are most important, in the cat the clasp and the dorsal and lateral aspects of the forelimb, the face area is large in the dog, and the hand and face in the monkey.

3. In the cat the digits are represented in two regions separated from the area for the face, but this appears to be an arrangement peculiar to one species and the triple representation suggested by the results of Marshall *et al.* has not been found.

4. Brief afferent volleys are sent to the cortex when the hairs are touched and more sustained discharges can be produced by pressure. With a wire electrode penetrating the grey matter impulses in single conducting units can be recorded. The pressure discharge in a single unit may last for a minute or more and the frequency shows some gradation according to the intensity of the stimulus. The impulses often appear in groups of two or three very closely spaced as in the discharges in the pyramidal tract.

5. The afferent volley produced by a touch is often followed by a rhythmic after-discharge from the thalamus. This has the same distribution to the cortex as the primary discharge. It consists of a series of short volleys at a frequency between 10 and 20 a second. It can be detected in the afferent fibres after destruction of the cortex and is therefore independent on a circuit from thalamus to cortex and back.

6. The arrival of an afferent volley produces a potential wave in the cortex with its first phase surface positive. This wave is due, as others have shown, to potential gradients in the afferent fibres and not to activity in the cortical neurones.

7. In chloralose anaesthesia the afferent cortical wave is followed by an efferent wave which is mainly surface negative and is associated with a discharge of impulses from the cortical neurones. When convulsant drugs are applied locally to the receiving area there is a similar afferent-efferent response to a touch, though the efferent component is much larger. The efferent response is abolished by restricting the blood supply to the cortex, etc.

8. With dial or nembutal anaesthesia in medium dosage there is motor activity in the cortex apart from stimulation and afferent discharges may have very little effect on this activity. The dissociation of the cortex from the afferent fibres is less marked when the cortex is less active and repeated tactile volleys usually succeed in imposing their rhythm on the cortical neurones.

9. In very deep dial or nembutal anaesthesia although the cortical neurones have become quite inactive afferent effects can still occur and rhythmic after-discharges from the thalamus will produce groups of surface positive cortical waves.

10. In deep anaesthesia with chloroform and ether afferent discharges may fail to reach the cortex. In lighter anaesthesia there are rapid potential oscillations which are increased in frequency by an afferent discharge. The cortical neurones are often well synchronized and may become more or less so during afferent excitation.

11. In light ether anaesthesia a touch may produce widespread activity in other parts of the cortex, though the rise of frequency is not as great as in the appropriate part of the receiving area. Auditory and visual stimulation may also produce a generalized increase in activity.

12. It appears that signals from tactile receptors are handed on to the cortex without much alteration, but that they are accompanied by additional signals from the thalamus which are much more variable.

13. The thalamic neurones resemble those of the cortex in their tendency to rhythmic discharge though they are more stable and less inclined to spontaneous activity. When the rhythmic activity of the brain is depressed (as in chloralose anaesthesia) it may react as though it were a simple reflex mechanism.

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THE GASEOUS TENSIONS IN THE BRAIN

BY F. C. COURTICE

From the Departments of Physiology and of Surgery, Oxford

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THE extracellular tension of gases has been determined by the injection of air into tissue spaces, and its recovery for analysis after it has reached equilibrium with the tissues around. Campbell [1931] has reviewed the experiments with the injection of air into the subcutaneous tissues, intestine, pleural and abdominal cavities. The carbon dioxide tension varied between 45 and 55 mm. mercury, while the oxygen tension varied between 20 and 40 mm. mercury. Campbell [1932] has also injected a bubble of air into the ventricles of the brain of rabbits and analysed this air 24 hr. later. He found that the carbon dioxide tension was 35-39 mm. and the oxygen tension 19-36 mm. mercury. From these experiments the oxygen tension of the brain tissue near the ventricles is approximately the same as or even lower than that in the other tissues investigated. This can be understood in view of the work of Lennox and his co-workers [1931, 1936] who showed that the percentage saturation of the venous blood coming from the brain was only 62, whereas the blood returning from the arm and leg, although not so constant, was less desaturated.

METHODS

In order to investigate further the tension of carbon dioxide and oxygen in the brain tissue, experiments have been performed in which air was injected into the ventricles of unanaesthetized human subjects after the withdrawal of cerebrospinal fluid, in the course of ventriculography. Samples of this air were withdrawn and analysed at varying intervals after injection, the subject lying at rest meantime. The samples of air were withdrawn by means of 10 or 20 c.c. syringes greased with paraffin and connected to the brain needle. The introduction of a three-way tap between the syringe and the needle enabled the pressure of air in the ventricle to be measured with the subject horizontal, one arm of the tap

being attached to a manometer. In several cases samples of arterial blood from the brachial artery and of venous blood from the jugular bulb were taken at the same time as the air was withdrawn. The percentage saturation, oxygen and carbon dioxide contents of these samples were determined. Carbon dioxide dissociation curves for oxygenated and reduced blood and the oxygen dissociation curve were in each individual case also determined. From all these determinations, the oxygen and carbon dioxide tensions of the arterial and venous blood of the brain were measured. The corresponding tensions in the air in the ventricle were estimated by ordinary gas analysis. All the human subjects were suffering from intracranial neoplasm, and in the majority of cases the ventricles were of greater capacity than normal.

Similar experiments were carried out in normal cats anaesthetized with chloralose. As the lateral ventricle of the cat is small, its exact position was first determined by measurement in a series of cats' brains. To tap the ventricle, the cat's head was held in a Palmer Stereotaxic machine. Bone over the ventricle was removed with a dental drill. Through this opening a needle was inserted into and held fixed in the cat's ventricle while cerebrospinal fluid was withdrawn and air injected. Only about 1 c.c. was injected and somewhat less was withdrawn. At the time of withdrawal arterial and sagittal sinus blood samples were taken as described in a previous paper [Courtice, 1940]. Determinations similar to those in man were made.

The air was analysed with the Haldane gas analysis apparatus, and the blood gases with the Haldane blood gas apparatus.

RESULTS

Fig. 1 shows in two human subjects the changes in the composition of the air introduced into the ventricular system of the brain. Both cases were suffering from hydrocephalus as the result of intracranial tumour, so that the capacity of the ventricles was in each case much greater than normal. The volume of cerebrospinal fluid withdrawn and of air introduced was 70 c.c. in the first case and 150 c.c. in the second. The time taken for the composition of the air to reach an equilibrium will depend to some extent upon the volume of air introduced. It can be seen that the carbon dioxide quickly reaches an equilibrium whereas the oxygen tension continues to fall for a considerable time. The oxygen tension is still falling after 3 and 5 hr. In two other cases samples were taken at intervals up to 24 hr. after the air had been injected. The oxygen tension fell in the first two hours roughly parallel with the two cases shown in

Fig. 1, and at the end of 24 hr. had reached a value of 40 mm. mercury in each case. The carbon dioxide tension rose rapidly in the first 1-2 hr. and then remained at a fairly constant level. From the experiments on human subjects, therefore, the carbon dioxide tension of the cerebrospinal fluid and of the brain tissue surrounding the ventricles can be quickly obtained, but the oxygen tension necessitates the air being left in the ventricles for at least 24 hr. Owing to the inadvisability of leaving air in the ventricles for 24 hr. or longer, it was not possible to obtain a large series of determinations of the oxygen tension in the ventricles.

The carbon dioxide tension has been studied in a series of twenty-one cases, samples of air being withdrawn from the ventricle about three hours after injection by which time the carbon dioxide has reached equilibrium. In eight of these cases samples of blood were taken from the brachial artery and from the jugular bulb just before the air was withdrawn from the ventricle. The tension of carbon dioxide was measured in the arterial and jugular blood as already described, while that in the air was determined by direct analysis. The results of these experiments are shown in Table 1. The mean tension of carbon dioxide in the twenty-

TABLE 1. CO₂ tension, mm. mercury

	Arterial blood	Jugular blood	Air in ventricle
	43	47	49
	35	44	44
	35	43	52
	37	47	45
	38	51	54
	35	48	45
	41	49	54
	35	44	51
Mean	37	47	49
			39
			51
			41
			50
			49
			43
			50
			47
			50
			50
			56
			50
			52
			<u>49</u>
		Mean of 21 cases	

one cases is 49 mm. mercury. In the group of cases in which blood samples were taken, the mean arterial CO₂ tension is 37 mm. mercury, jugular blood 47 mm. mercury and in the ventricles 49 mm. mercury. Measured

in this way the CO_2 tension in the tissue surrounding the ventricles is on the whole slightly higher than that in the jugular blood and very much higher than that in the arterial blood.

The oxygen tension of the air in the ventricles had not reached equilibrium in the above group of cases, but in the two cases in which air remained in the ventricles for 24 hr. the oxygen tension was in each case 40 mm. mercury. In both of these cases the ventricles were enlarged, but although about 100 to 200 c.c. of air were introduced, the greater part of it was absorbed within 24 hr. as seen by X-ray. Thus it seems quite likely that since only a small volume of air remained, gaseous equilibrium had been reached.

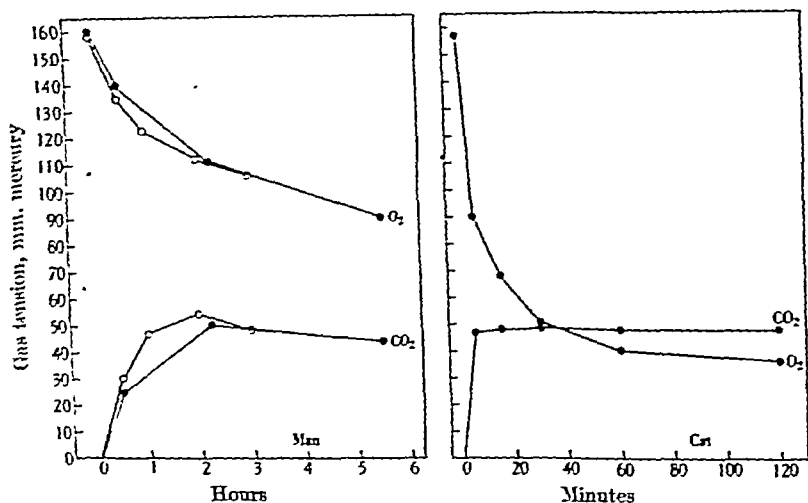


Fig. 1. The changes in the gaseous tensions of air introduced into the ventricles of man and of cats.

With cats the ventricles are of course very small, and the volume of air introduced was approximately only 1 c.c., a much smaller volume than in human subjects. It was found that the tensions of carbon dioxide and of oxygen therefore reached equilibrium in a much shorter time. Fig. 1 shows the mean of three experiments, each individual experiment giving practically identical results. The carbon dioxide tension again reaches a steady level very quickly, while the oxygen tension takes much longer. In a group of five cats, the oxygen and carbon dioxide tensions of the arterial and sagittal sinus blood have been determined together with the tensions of these gases in the air introduced into the ventricle. The results are shown in Table 2. The carbon dioxide tension behaves

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	35	48	45
	41	49	54
	35	44	51
Mean	37	47	49
			39
			51
			41
			50
			49
			43
			50
			47
			50
			50
			56
			50
			52
			49
		Mean of 21 cases	

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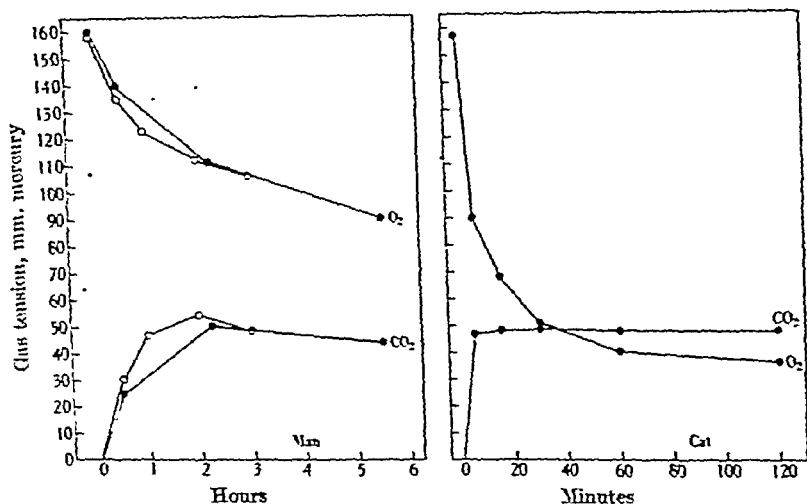


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TABLE 2

CO ₂ tension, mm. mercury			O ₂ tension, mm. mercury	
Arterial blood	Sinus blood	Air in ventricle	Sinus blood	Air in ventricle
39	47	46	46	40
44	52	51	40	30
38	46	48	44	38
41	53	51	43	33
36	46	47	37	24
40	49	49	42	33

very much the same as in the human cases, the tension in the ventricle being the same as in the sinus blood. The oxygen tension of the air in the ventricle was, however, somewhat lower than that of the sinus blood.

In all the above experiments the cats were breathing room air, and the arterial blood was in all cases about 95 % saturated with oxygen. In three experiments, air was introduced into the ventricle of the cat and was withdrawn after the cat had been breathing air containing 10 % oxygen for 2 hr. The results are shown in Table 3. It can be seen that

TABLE 3. Oxygen tension, mm. mercury

	Sinus blood	Air in ventricle
	16	16
	22	21
	26	19
Mean	21	19

when there is less oxygen available the tension in the tissues falls and is always somewhat less than the tension in the venous blood coming from the brain.

DISCUSSION

When cerebrospinal fluid of the ventricles is replaced by air in man and in the cat carbon dioxide rapidly diffuses into this air and oxygen more slowly diffuses out, so that for a time the combined tensions of oxygen and of carbon dioxide are greater than those of the air injected. The carbon dioxide probably comes partly from any remaining cerebrospinal fluid and partly from the surrounding brain tissue. In Table 4 are shown the bicarbonate content of arterial and jugular plasma and of cerebrospinal fluid in a series of human subjects. In some cases the cerebrospinal fluid (taken from the ventricles in all cases) has a bicarbonate content approximately equal to that of the venous plasma while in others it approximates that of the arterial plasma. In any event, the cerebrospinal fluid has a high bicarbonate content, and with a tension of 49 mm. mercury it can easily be understood how rapidly carbon dioxide diffuses into the injected air.

TABLE 4. Bicarbonate content, c.c. CO₂%

Arterial plasma	Venous plasma	Cerebrospinal fluid
48.4	56.7	57.1
56.9	67.5	62.0
50.6	55.6	55.5
54.4	60.0	58.6
66.6	73.4	66.5
68.4	72.9	67.7
52.8	60.6	59.5
59.3	69.7	63.2
60.7	67.2	61.1
60.5	69.6	60.6
55.7	61.5	59.4
57.6	64.2	55.5
Mean 57.7	64.9	60.6

It is reasonable to suppose that the ventricular cerebrospinal fluid and the tissues in the immediate neighbourhood are in gaseous equilibrium and that the gas tensions in the air when equilibrium has been reached are the same as those in the cerebrospinal fluid. The formation of cerebrospinal fluid by the choroid plexuses is at present held by many to be by secretion rather than by simple diffusion, so that the tension of carbon dioxide in the cerebrospinal fluid may not be the same as that in the capillaries of the choroid plexuses.

The tensions of oxygen and of carbon dioxide in the ventricular cerebrospinal fluid and surrounding tissues show a fairly close relationship to the corresponding tensions in the venous blood leaving the brain. This venous blood represents the blood coming from the brain as a whole, and probably varies in gaseous composition in different parts of the brain. Nevertheless, it seems that a better idea of the tensions of oxygen and of carbon dioxide in the brain tissues can be gained by the determination of these tensions in the blood coming from the brain than in the arterial blood or the alveolar air. This is especially so when the cerebral circulation is increased by an alteration in the gaseous composition of the inspired air, as will be seen in a later paper.

I am indebted to Prof. Hugh Cairns for his help in the experiments on human subjects.

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THE EFFECT OF OXYGEN LACK ON THE CEREBRAL CIRCULATION

By F. C. COURTICE

From the Departments of Physiology and of Surgery, Oxford

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BRAIN tissue cannot survive for long without an adequate supply of oxygen. A decreasing oxygen tension in the inspired air will ultimately produce unconsciousness and death due to an insufficient supply of oxygen to the brain. However, although the brain is so sensitive to a lack of oxygen, the normal oxygen uptake from the blood passing through the brain of human subjects at rest is greater than that passing through the leg or arm [Lennox & Leonhardt, 1931]. The percentage saturation of the haemoglobin of the blood coming from the brain is approximately 60, whereas the percentage saturation of the haemoglobin of the blood coming from the arm and leg at rest is greater than this. This means that the oxygen tension in the venous blood of the brain is lower than that in the venous blood of the leg or arm. A percentage saturation of 60 corresponds on the oxygen dissociation curve in human subjects to a tension of only 36 mm. mercury. This seems remarkable in an organ which is so sensitive to decreased oxygen tension. Lennox, Gibbs & Gibbs [1935] have also shown that in human subjects unconsciousness occurs when the percentage saturation of the haemoglobin of the jugular blood has been reduced to about 25 (although Courtice [1940*a*] obtained lower figures without loss of consciousness, after raising intracranial pressure) which corresponds on the oxygen dissociation curve to a tension of 19 mm. mercury. It is probable that the oxygen tension in the brain tissue is slightly lower than these tensions in the venous blood [Courtice, 1941], but the actual fall in the oxygen tension of the brain tissue which will produce unconsciousness will be approximately from 36 to 19 mm. mercury. Although the resting oxygen tension in the limb muscles is greater than that of the brain, very low oxygen tensions may be reached in severe muscular exercise without causing any damage to the muscle tissue, whereas such

low tensions in the brain would cause permanent damage if not death [cf. Wolff, 1936].

An increase in the carbon dioxide tension of the blood causes an increased cerebral circulation [Wolff, 1936] by dilatation of the cerebral vessels, thus tending to check the increase of carbon dioxide tension in the brain. Since the fall in oxygen tension in the jugular blood from normal to that producing unconsciousness is not very great, it would seem that if the tension of oxygen in the brain began to fall, compensatory mechanisms would immediately be brought into action to prevent the oxygen tension falling further.

In order to find how the tension of oxygen in the brain varies, and what compensatory mechanisms are involved when the oxygen tension in the inspired air is lowered, experiments were performed with cats anaesthetized with chloralose (85 mg./kg. body weight, intravenously). It has been shown [Courtice, 1940*a*] that in cats under chloralose anaesthesia the percentage saturation of the haemoglobin of the arterial blood and of the blood coming from the brain was very constant and closely resembled the figures obtained in normal unanaesthetized human subjects. Moreover, Chute & Smyth [1939] showed that chloralose did not affect the oxygen uptake by the perfused cat's brain.

Samples of blood were taken from the femoral artery and torcula simultaneously as described previously [Courtice, 1940*a*]. The percentage saturation, oxygen content, carbon dioxide content and lactic acid content were estimated in the blood samples, the Haldane blood gas apparatus being used for the blood gases, and the Friedemann, Cotonio & Shaffer [1927] method for the lactic acid. From the arterio-venous oxygen difference a measure of the cerebral circulation rate was obtained. Blood pressure was recorded from the femoral artery on a smoked drum, and respiration was recorded with a Palmer respiration recorder, the rate, depth and volume of respiration thus being recorded. The cats were allowed to breathe mixtures of oxygen and nitrogen from Douglas bags. Blood samples were taken at intervals, while the blood pressure and respiration were recorded continuously.

RESULTS

In the first group of experiments, a series of oxygen and nitrogen mixtures were breathed by the cat. Normal readings were first obtained with the cat breathing room air, and then Douglas bags containing 18, 15, 12, 9 and 6% oxygen in nitrogen were applied to the Palmer respiration apparatus, each for 5-8 min. As soon as one bag was removed the next

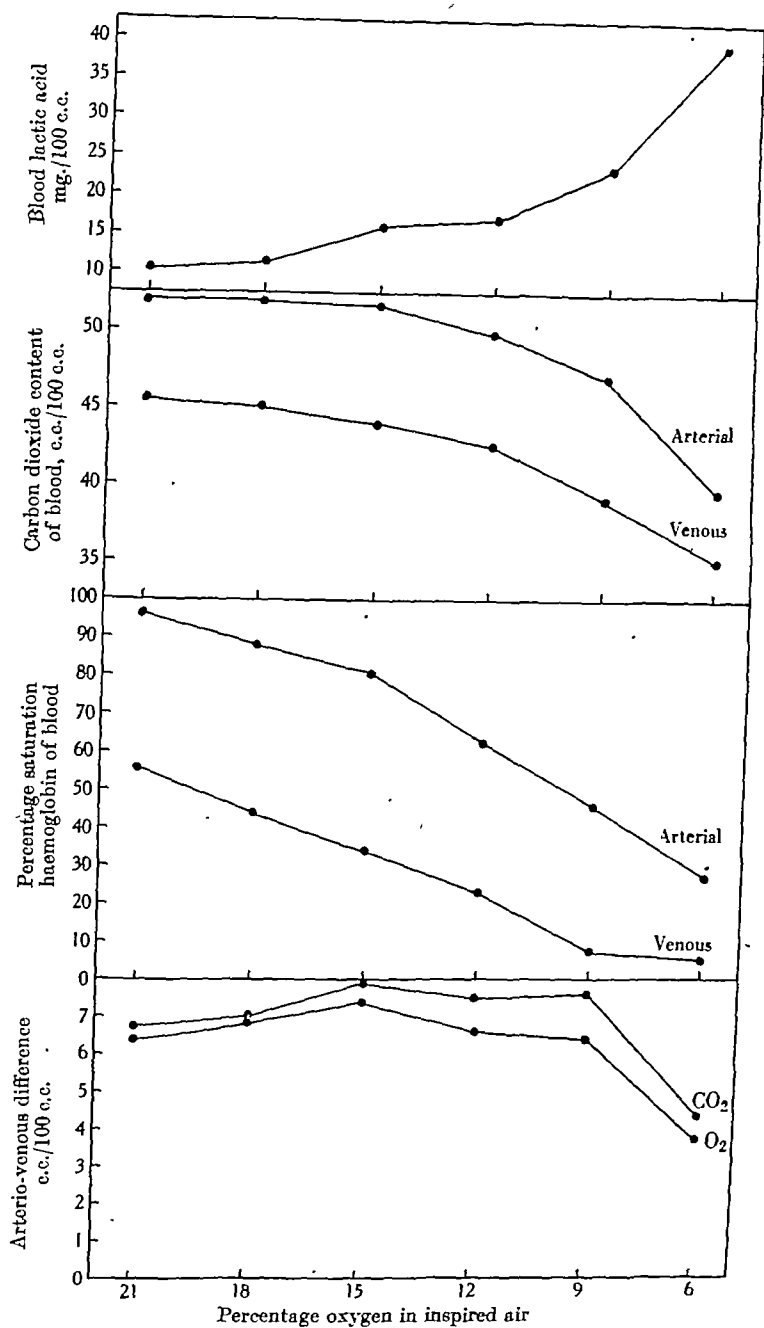


Fig. 1. The effects of breathing air containing 21, 18, 15, 12, 9 and 6% oxygen on the arterio-venous oxygen and carbon dioxide differences, percentage saturation of the arterial and sinus blood, arterial and sinus carbon dioxide contents and the blood lactic

was applied. In this way the cat breathed 21, 18, 15, 12, 9, and 6% oxygen for successive periods, the blood samples being taken at the end of each period just before the bags were changed.

Almost identical results were obtained in each of four animals, and the results of a typical experiment are shown in Figs. 1 and 2. It can be

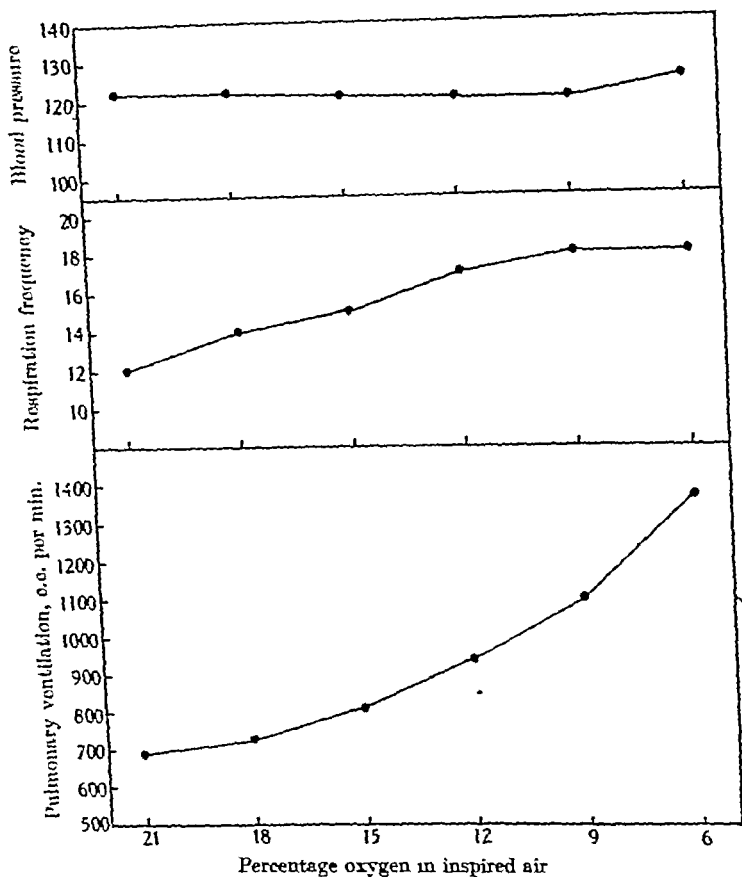


Fig. 2. The effects of breathing air containing 21, 18, 15, 12, 9 and 6% oxygen on the total pulmonary ventilation, respiration frequency and blood pressure.

seen that at first as the tension of oxygen in the inspired air is decreased, the percentage saturation of the blood coming from the brain decreases at about the same rate as that of the arterial blood. The arterio-venous oxygen difference accordingly remains fairly constant. Provided the metabolism of the brain tissue remains constant, therefore, the cerebral blood flow remains constant. This is so while the air breathed contains

21 to about 12% oxygen. It seems probable that the tension of oxygen in the brain tissue is not very different from that in the blood coming from the brain. Therefore, within these limits there appears to be no marked increase in the cerebral circulation to compensate for the falling oxygen tension in the brain.

When 12% and lower percentages of oxygen are breathed, the arterio-venous oxygen difference begins to decrease, while when only 6% of oxygen is breathed this difference is markedly decreased, which suggests that the cerebral circulation is in this case considerably speeded up in an endeavour to prevent the oxygen tension falling too much, although when the inspired air contains only 6% of oxygen the oxygen tension in the blood coming from the brain is very low and could not fall much farther.

In control experiments, the percentage saturation of the arterial and sinus blood remained very constant for an hour in each of four animals. The mean results of these four experiments are shown in Table 1.

TABLE 1

	Oxygen capacity c.c./100 c.c.	Percentage saturation		A.-v. oxygen difference c.c./100 c.c.
		Arterial	Sinus	
First sample	14.47	96	60	5.4
1 hr. later	14.37	96	60	5.3

Associated with the fall in oxygen tension in the brain tissue when air deficient in oxygen is breathed, are changes in lactic acid content of the blood. It can be seen from Fig. 1 that as the oxygen tension in the sinus blood and thus in the brain tissue falls the lactic acid rises, very gradually at first but more rapidly when 9 and 6% of oxygen are breathed. The blood lactic acid reached a value of 40 mg./100 c.c. after the animal had been breathing 6% oxygen for only 8 min. In this short time the lactic acid content of the blood and tissues has probably not come into equilibrium, so that the lactic acid content of the latter may be greater than this amount.

With the rise in lactic acid, a corresponding fall in the blood carbon dioxide content occurred as can be seen in Fig. 1. At first there is practically no change in the carbon dioxide content, but with lower oxygen tensions the carbon dioxide content falls more rapidly as the lactic acid rises.

It seems that the rise in the lactic acid content of the blood, by virtue of its acidity, probably plays some part in dilating the cerebral vessels, thus causing an increased blood flow through the brain. Another factor

which will increase the blood flow is a rise in the blood pressure. Dilatation of the blood vessels will not increase the cerebral blood flow unless the blood pressure is maintained or raised. In Fig. 2 is plotted the mean blood pressure measured from the tracings obtained. When the oxygen in the inspired air was gradually reduced as in this group of experiments, the rise of blood pressure was not marked, although in some cases it rose when the lower percentages of oxygen were breathed.

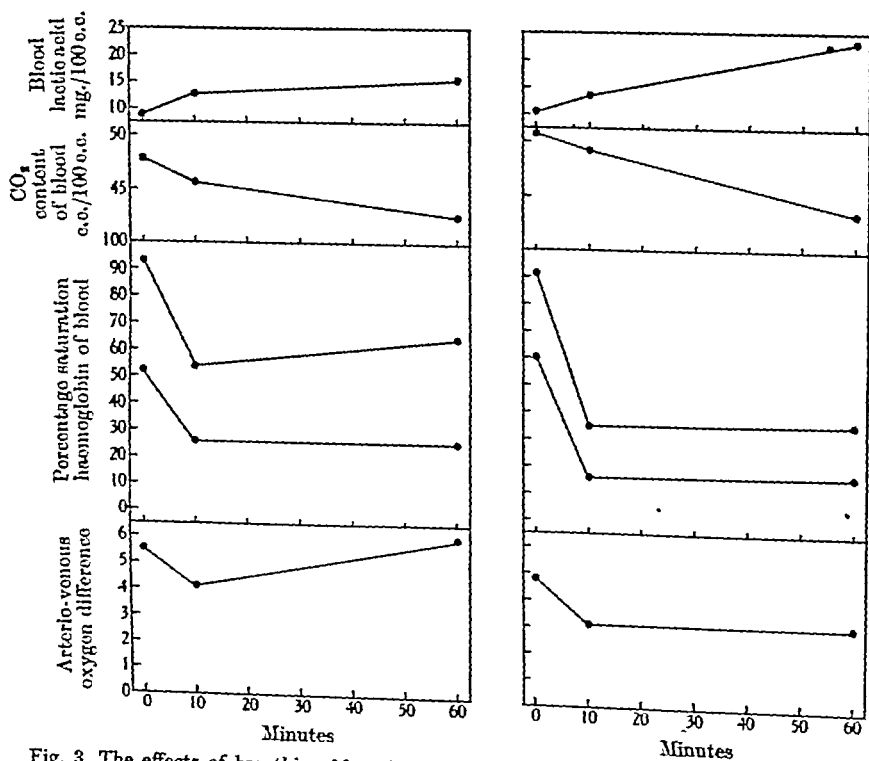


Fig. 3. The effects of breathing 12 and 10% oxygen respectively on the arterio-venous oxygen difference, percentage saturation of the arterial and sinus blood, carbon dioxide and lactic acid contents of arterial blood. Left: 12% oxygen begun at zero. Right: 10% oxygen begun at zero.

The increased cerebral blood flow with severe oxygen lack constitutes an important compensatory mechanism in checking the fall of oxygen tension in the brain tissue. Another mechanism is the increase in pulmonary ventilation. In Fig. 2 is shown the effect of oxygen lack on the frequency of breathing and on the total pulmonary ventilation. These measurements are the mean values obtained from the tracing for each period of breathing the various oxygen nitrogen mixtures. The total

pulmonary ventilation increases very little with 18 and 15% oxygen, but much more rapidly as the tension of oxygen in the inspired air further falls. This increase in respiration is again coincident with the rise of lactic acid in the blood.

The above experiments show the changes which take place when the oxygen lack is somewhat gradually applied. Figs. 3 and 4 show the changes when mixtures of air containing 12 and 10% oxygen are breathed for one hour, the oxygen content of the inspired air being suddenly changed from 21 to 12 and 10% respectively.

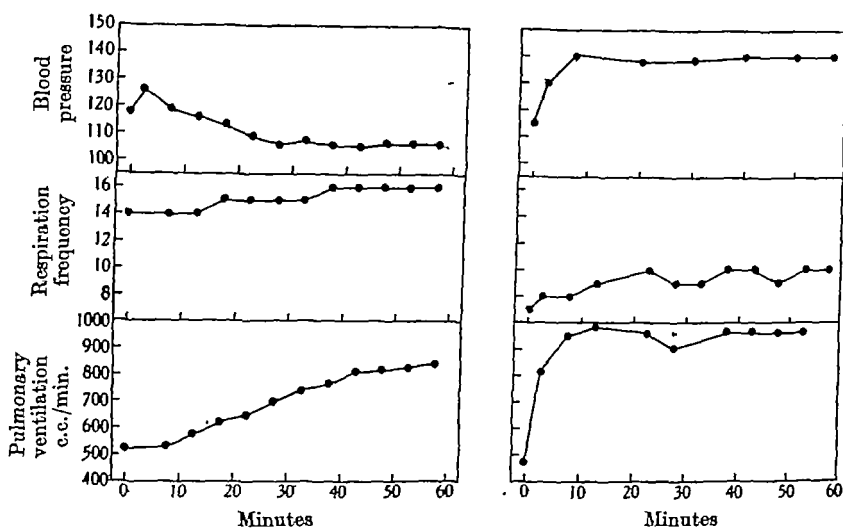


Fig. 4. The effects of breathing 12 and 10% oxygen on the total pulmonary ventilation, respiration frequency and blood pressure. Left: 12% oxygen. Right: 10% oxygen.

When the oxygen content of the inspired air is suddenly reduced from 21 to 12%, the percentage saturation of the arterial and sinus blood both fall rapidly, but the arterio-venous oxygen difference decreases which suggests that the cerebral blood flow is increased. The percentage saturation of the haemoglobin of the arterial blood fell from 93 to 54 at the end of 10 min., but after the mixture had been breathed for 1 hr. the haemoglobin saturation of the arterial blood had risen to 65, while the arterio-venous oxygen difference also rose. This rise in the arterial haemoglobin saturation is the result of an increase in the pulmonary ventilation which increases the alveolar oxygen tension. In Fig. 4 it can be seen that when 12% oxygen is breathed, the pulmonary ventilation gradually increases, while the blood pressure shows a short-lived rise. The lactic acid content

rose gradually from 8.8 to 15.7 mg./100 c.c. in the hour, while the carbon dioxide content gradually fell.

When 10% oxygen was breathed, the arterial percentage saturation of the haemoglobin fell from 91 to 35 in 10 min. and after 1 hr. it was 36. The percentage saturation of the haemoglobin of the sinus blood also fell

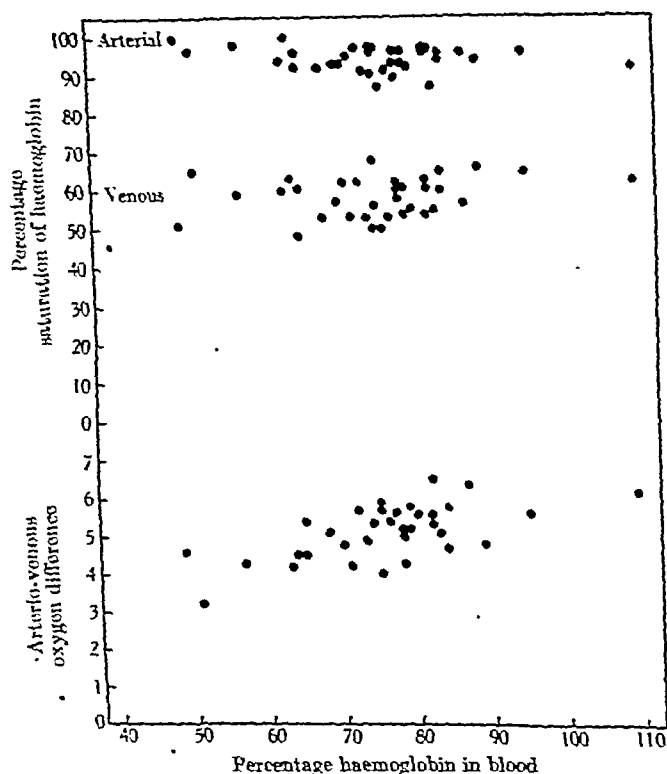


Fig. 5. The relation between the haemoglobin content of the blood and the percentage saturation of arterial and sinus blood and the arterio-venous oxygen difference in thirty-five normal cats.

markedly, while the arterio-venous oxygen difference decreased and remained at this low level for an hour. The pulmonary ventilation rose rapidly in the first 10 min. and remained constant for an hour. This constant pulmonary ventilation agrees with the constancy of the percentage saturation of the arterial and sinus blood. The blood pressure also rose rapidly and remained at a fairly steady level for an hour. The lactic acid content of the blood rose from 10 to 24 mg./100 c.c., while the carbon dioxide content fell accordingly.

These experiments show the behaviour of the oxygen tension in the blood entering and leaving the brain when an animal is suffering from acute oxygen lack, resulting from a diminished oxygen tension in the inspired air at normal atmospheric pressure. It has been seen that the oxygen tension and the oxygen content of both arterial and sinus blood may fall considerably before any increase in cerebral circulation occurs. No prolonged exposures to oxygen want have been studied to ascertain the changes that occur when acclimatization has occurred. When, however, the oxygen content of the arterial blood is decreased by a lowered haemoglobin content of the blood, and not by a lowered oxygen tension of the arterial blood, somewhat different results are probably obtained. In Fig. 5 are plotted the percentage saturation of the arterial and sinus blood and the arterio-venous oxygen difference against the haemoglobin content (calculated as percentage of a normal value of 18.5 c.c. oxygen capacity per 100 c.c. blood) in a series of thirty-five normal cats. These results are not conclusive, but it appears that the percentage saturation of the arterial and sinus blood remains fairly constant, while the arterio-venous oxygen difference falls as the oxygen capacity of the blood falls. This would mean that the tension of oxygen in a normal cat's brain remains constant even though the oxygen content of the blood entering the brain may fall, the tension in the brain being kept normal by an increase in the blood flow. Yet in mild acute oxygen lack (21 to 15% oxygen in the inspired air), there is no apparent tendency to prevent the oxygen tension of the brain tissues from falling. It is possible that in very prolonged exposures to these percentages of oxygen, compensatory mechanisms will gradually raise the oxygen tension by a gradual increase in the cerebral circulation. In more severe oxygen lack, this mechanism is evident immediately.

DISCUSSION

The estimation of the cerebral blood flow by determination of the arterio-venous oxygen and carbon dioxide differences depends on the constancy of the brain metabolism. If this remains constant, a fall in the arterio-venous oxygen difference will indicate a rise in the cerebral circulation rate. It is probable that with a moderate degree of oxygen lack the metabolism of the brain will not vary, but when more severe oxygen lack is observed such as breathing 9 and 6% oxygen, this may not be so. The results therefore show that when the inspired air contains 21 to 15% oxygen, there is no increase in the cerebral circulation in spite of a considerable fall in the oxygen tension in the venous blood coming from the brain. In Fig. 6 are plotted the oxygen tensions in the inspired

air, arterial blood and sinus blood when 21 to 6% oxygen mixtures are breathed. The tensions of oxygen in the arterial and sinus blood have been determined from the mean dissociation curve of the blood of several cats.

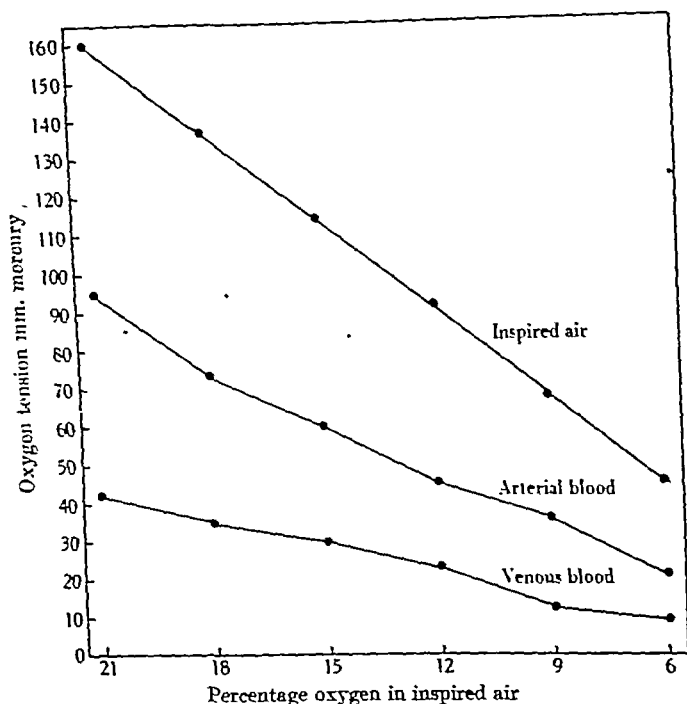


Fig. 6. The tensions of oxygen in the inspired air, arterial and sinus blood when the percentage of oxygen in the inspired air is decreased.

From this diagram it can be seen that the oxygen tension in the sinus blood may fall from 42 to 30 mm. mercury, without causing any compensatory increase in the cerebral circulation as seen in Fig. 1. When 12 and lower percentages of oxygen are breathed the arterio-venous oxygen difference begins to fall. Thus when the tension of oxygen in the sagittal sinus blood falls below 30 mm. mercury the cerebral circulation is increased.

This increase in cerebral circulation is coincident with a rise in the blood lactic acid. The cause of the rise of lactic acid in the blood with decreasing oxygen tension is probably due to incomplete oxidation of carbohydrate, and maybe also to a secretion of adrenaline which will increase the blood lactic acid, the exact mechanism of which is unknown. The site of lactic acid formation after adrenaline is the muscle glycogen [Cori, 1931], but in human subjects adrenaline causes a rapid increase

in pulmonary ventilation which decreases again before the blood lactic acid has reached its maximum [Courtice, Douglas & Priestley, 1939]. This suggests that lactic acid is also produced *in situ* in the brain tissue. As the brain utilizes carbohydrate only [Lennox & Leonhardt, 1931; Courtice, 1940*b*, etc.] it seems that in conditions of oxygen lack lactic acid might be formed in the brain tissue as well as elsewhere. In Fig. 1, as well as in all experiments in this series, the arterio-venous carbon dioxide difference is approximately equal to the arterio-venous oxygen difference while 21, 18 and 15 % oxygen are breathed, but with more severe oxygen lack when the lactic acid of the blood rises, the arterio-venous carbon dioxide difference is greater than the arterio-venous oxygen difference. This suggests that lactic acid formed in the tissues of the brain is expelling carbon dioxide other than that resulting from oxidation. The rise of lactic acid in the brain tissue may therefore in short experiments be greater than the rise in the blood as equilibrium will not have been reached. Thus when animals are exposed to severe oxygen want for a short time, it seems probable that a sudden rise in the lactic acid in the brain tissue will increase respiration and cerebral circulation before the blood lactic acid rises very much.

The effects of an increase in the lactic acid content of the brain tissue will be to dilate the cerebral vessels [cf. Wolff, 1936]. Provided the blood pressure does not fall, this dilatation of the cerebral vessels will increase the cerebral blood flow. It has been seen that with moderate degrees of oxygen lack the blood pressure remains fairly constant (Fig. 2), while with more severe oxygen lack the blood pressure rises (Fig. 4). Therefore, the combined effects of an increase in lactic acid and an increase in blood pressure will cause an increase in the cerebral blood flow. From the experiments described above, it seems that a fall in oxygen tension in the brain does not increase the cerebral circulation until the lactic acid content of the blood and the blood pressure begin to rise.

Previous workers have shown an increased cerebral blood flow with severe oxygen lack. Lennox & Leonhardt [1932] showed in man that the arterio-venous oxygen difference of the brain fell with pronounced anoxaemia. Gibbs, Gibbs & Lennox [1935], using the thermocouple in the jugular bulb in man, showed that in subjects breathing nitrogen the cerebral blood flow increased; this, however, is rather a severe form of anoxaemia. Schmidt & Pierson [1934] have also found that low oxygen tensions increase the blood flow in the medulla and hypothalamus of the cat. They used a thermocouple which was introduced into the brain tissue.

The effects of oxygen lack on respiration are well known. In human subjects there is little increase in respiration while the oxygen in the inspired air falls from 21 to 14%, but with percentages lower than this pulmonary ventilation increases [Haldane & Priestley, 1936]. In the experiments described above, the pulmonary ventilation increased, as shown in Fig. 2, with a decrease in the oxygen tension in the inspired air. This increase in pulmonary ventilation is slight in moderate anoxaemia and much more rapid as the oxygen lack becomes more severe. The rise in pulmonary ventilation runs parallel with the rise in blood lactic acid. In recent years much work has been done on the effects of anoxaemia on respiration. This work has been reviewed recently by Schmidt & Comroe [1940]. The evidence indicates that oxygen lack increases pulmonary ventilation mainly by reflex action through the carotid sinus-carotid body mechanism, whereas increased carbon dioxide tension acts mainly on the respiratory centre. Schmidt & Comroe have also obtained evidence that an increase in the hydrogen ion concentration of the blood increases pulmonary ventilation by means of the carotid sinus reflex. Thus it seems possible that anoxaemia will increase pulmonary ventilation by a rise in lactic acid acting peripherally on the carotid sinus or body as suggested by Gesell [1939]. If, however, carbon dioxide acts centrally by virtue of a change in the pH, it seems that a rise of lactic acid in the cells of the brain tissue will also act centrally by virtue of a rise in the hydrogen ion concentration. Nevertheless, wherever the site of action may be, it seems in these experiments that the rise in lactic acid must play some part in increasing the pulmonary ventilation when air deficient in oxygen is breathed.

The effects of the changes in pulmonary ventilation on the tension of oxygen in the arterial blood are shown in Fig. 6. When 18% oxygen is breathed the difference in oxygen tension between inspired air and arterial blood is the same as when 21% oxygen is breathed since there is practically no change in pulmonary ventilation (Fig. 2). As the oxygen in the inspired air decreases and the pulmonary ventilation increases, so this difference between the oxygen tension in the inspired air and in the arterial blood naturally decreases. This increase in pulmonary ventilation will thus prevent the oxygen tension in the arterial blood from falling too rapidly although this mechanism is very slow in starting.

Thus two of the main mechanisms, an increased pulmonary ventilation and an increased cerebral circulation, tending to check the fall in oxygen tension in the brain tissue begin to act when the inspired air contains less than about 15% oxygen. These compensatory mechanisms

are gradual at first and increase very rapidly as the anoxaemia becomes more severe.

The only mechanism which prevents the oxygen tension in the brain from falling too rapidly in moderate degrees of oxygen lack is the S-shape of the oxygen dissociation curve. This can be seen in Fig. 6. While the oxygen in the inspired air falls from 21 to 15 %, the arterio-venous oxygen difference and hence the cerebral blood flow remain fairly constant, but owing to the S-shape of the oxygen dissociation curve the tension of oxygen in the sinus blood does not fall as rapidly as that in the arterial blood.

When the oxygen content of the blood going to the brain was decreased, not by a decreased oxygen tension but by a decreased haemoglobin content, it seems that the tension of oxygen in the brain is kept constant by an increase in the cerebral circulation (Fig. 5). This increase in cerebral circulation may be due partly to a decrease in the viscosity of the blood when the red cell content decreases. However, further experiments are necessary before any definite conclusions can be made. Owing to the outbreak of the war these experiments have had to be postponed.

SUMMARY

The cerebral circulation as measured by the arterio-venous oxygen differences between the arterial and the sinus blood, the lactic acid content of the blood, the blood pressure and the pulmonary ventilation have been determined in chloralosed cats exposed to varying degrees of oxygen lack.

The cerebral circulation and the pulmonary ventilation do not increase until the inspired air contains less than about 15% oxygen. With lower oxygen tensions they increase, gradually at first and then more rapidly. Associated with these changes is a rise in the blood lactic acid content.

The cerebral circulation with more severe degrees of anoxaemia is increased partly by a rise in blood pressure and partly by a dilatation of the cerebral vessels caused by the rise in lactic acid.

The tension of oxygen in the brain tissue as judged by the tension in the sinus blood falls rapidly with decreasing oxygen tension in the inspired air, the chief mechanisms preventing this fall from being greater being the S-shape of the oxygen dissociation curve in moderate anoxaemia, and an increase in pulmonary ventilation and cerebral circulation in more severe anoxaemia.

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THE PHOTO-SENSITIZING ACTION OF BUCKWHEAT
(*FAGOPYRUM ESCULENTUM*)

BY HARRIETTE CHICK AND PHILIPP ELLINGER

*From the Division of Nutrition, Lister Institute and
Roebuck House, Cambridge*

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BUCKWHEAT, *Fagopyrum* (nat. order Polygonaceae), has been known in Europe for at least four centuries as a food for human beings and animals [Hehn, 1902], which occasionally gives rise in the latter to a disease known as fagopyrism. This occurs in sheep, pigs, cattle, goats and horses, and has been produced experimentally in rabbits, guinea-pigs, mice and rats. Only albino or partially pigmented animals are affected and exposure to light is necessary. The symptoms include erythema, with itching, of the less pigmented parts of the skin, which may be followed by roughness of the fur with loss of hair; eventually necrosis of areas of the skin may occur. Similar changes have been observed in the mucous membranes, and in severer cases the nervous system may also be affected, and convulsions with paralysis of the limbs and respiratory muscles may cause death. Hertwig [1833] first recognized the connexion of the disease with exposure to sunlight of the unpigmented parts of the skin and Darwin [1868] also discussed the immunity of pigmented animals.

Observations of naturally occurring buckwheat poisoning have been made chiefly by veterinary surgeons on farm animals invading fields of buckwheat or stall-fed on cut buckwheat or buckwheat seeds, or bedded on buckwheat straw. The green plant, the flowers, seeds, straw, stubble and chaff have all been considered dangerous by different authors. Descriptions of this work are to be found in the papers of Merian [1915] and Lutz & Schmid [1930].

In laboratory studies, in which various parts of the plant were fed separately, Oehmke [1908] found both meal and bran active, Fischer [1909] the bran active but the meal inactive; Merian [1915] found activity in both green and flowering shoots, Sheard, Caylor & Schlotthauer [1928]

in the green plant and seeds and Lutz & Schmid [1930] in the flowering plant and the seeds, while Bichlmaier [1912] and Hilz [1913] were unable to produce photosensitization with any part of the plant.

The fact that buckwheat meal, from which the husk of the seed is removed in ordinary milling, is regularly consumed with no disadvantage by men and animals is a strong indication that the poisonous substance is not present in the kernel of the seed. This is confirmed by the results of experiments described in the present paper.

Many theories have been put forward for the aetiology of the disease, mostly without adequate evidence. They have been fully reviewed by Merian [1915] and Lutz & Schmid [1930] and need not be mentioned here. The role played by light in buckwheat disease has been amply confirmed by experiments in which portions of the skin of sensitive animals have been protected from light [Wedding, Ascherson & Virchow, 1887], and different sources of light have been investigated [Merian, 1915; Sheard *et al.* 1928; and Lutz & Schmid, 1930]. Reports on the effect of these different sources have not always been in agreement and too much reliance should not be placed on them, unless the distribution in the different parts of the spectrum of the intensity of the beam is also reported. Differing intensity in the active region of the source of light used will explain many contradictions in the literature.

The aim of the present work was to investigate the distribution of the activating substance in the different parts of the buckwheat plant, to study the mechanism of sensitization in the animal and to determine to which portion of the spectrum the active rays belonged. Preliminary attempts to isolate the activating pigment from buckwheat flowers, of which a note has already been published [Chick & Ellinger, 1940], are also described.

PART I. DETERMINATION OF THE ACTIVE PORTION OF THE BUCKWHEAT PLANT, OF THE MINIMUM ACTIVATING DOSE AND OF THE ACTIVE WAVE-LENGTH

METHODS

Ordinary buckwheat, *Fagopyrum esculentum*, was used. The seeds as purchased from the local corn merchant were examined, and crops were also specially grown at Branscombe, Devon, and at Cambridge, so that the leaves and shoots of the growing plant and the flowers at different stages of maturity might be collected separately and investigated. The different materials were dried in a domestic airing cupboard or in an incubator room at 37° C. The dried buckwheat flowers were usually

powdered in a mortar as finely as possible, dried again to constant weight in a vacuum desiccator over sulphuric acid and kept in tins in a refrigerator room. The photosensitizing activity of material preserved in this manner remained unchanged for 2 years.

Animals and diets

Young albino rats obtained from various sources were used and a few tests were made with mice from the stock of the Lister Institute at Elstree. On the whole the rat was found to be far more satisfactory.

In feeding the seeds, a diet was prepared containing: ground buckwheat seed 90 parts, hardened cotton-seed oil 5, lard 2, salt (McCollum's no. 185) 2, with a few drops of cod-liver oil given daily to each animal separately, to provide vitamins A and D. The diet was calculated to contain about 9.6% of protein derived entirely from the buckwheat. The grains were ground in a small electrically driven steel mill in the laboratory. The husks, being very hard, came through the mill almost unbroken and could readily be removed by sieving. When the entire meal was put in the diet, the husks remained uneaten and, in order to secure that the whole grain was consumed, the following method was used. Before grinding, the grains were soaked in water and kept in a hot room at 37° C. for about 8 hr.; the quantity of water was adjusted to the amount which could be completely absorbed by the grain and contributed about 80% of the weight of the diet when made. The softened grain and husk, when subsequently milled, yielded a homogeneous product and, when this was incorporated in the diet, the whole was eaten. To prevent scattering, the diets were mixed with water to form a thick pasty cream before being fed.

When examining other parts of the buckwheat plant, a diet similar to the above was used, in which the buckwheat was replaced by ground whole wheat. It contained: whole ground wheat 93 parts, casein 3, hardened cotton-seed oil 5, salt mixture 1.5, with a few drops of cod-liver oil to each animal daily. Weighed amounts of the dried, powdered, plant materials, given either in small daily doses or in single larger doses, were added to the diet and thoroughly mixed with it both before and after addition of the water. The amount of diet given was adjusted to that which could be entirely consumed. Control rats received the wheat diet without buckwheat addition or a third diet in which ground whole maize¹ replaced the wheat.

¹ The maize was a specimen kindly sent by Dr T. D. Spies from a district in the southern United States where pellagra is endemic. Animals fed on this maize showed no sensitiveness to light, thus confirming a long series of results obtained in this laboratory with rats and mice fed on maize. In no case has any sensitiveness to light been observed.

In other experiments, designed to find the minimum sensitizing dose of the dried flowers, the animals received a synthetic diet consisting of 'light white' casein 10 parts, hardened cotton-seed oil 3.5, rice starch 30, lard 5, dried yeast 10, cod-liver oil 1, and water 50. This formed a dough-like mixture which could be rolled into balls and conveniently fed, the dose of powdered flowers being mixed in a small portion and thus fed.

The rats thrive on these diets, but especially on that containing 90% of buckwheat meal; on this, reproduction took place and a second generation was reared, a surprising result in view of the low proportion of protein, only 9.6%.

Sensitivity to light

This was tested either by exposure of the whole animal to sunlight or to a 1500 W. lamp, or of a circumscribed portion of the skin, such as one ear or a shaved area of the skin of the back, to local irradiation in the beam of other artificial sources of light, e.g. carbon-arc or mercury-vapour lamp.

After exposure of the whole animal to light, the following symptoms developed in a sensitized animal and were regarded as evidence of a positive effect. The animals became irritable, scratched themselves and were obviously uncomfortable. The portions of the skin uncovered by fur, the ears, nose, tail and paws, became swollen and inflamed and remained so for some time after the animals were removed from the light source. If the exposures were long, 5-6 hr., convulsive fits sometimes occurred, especially if the exposures were repeated at short intervals. Such intense reactions were accompanied by temporary loss of appetite and weight. On two occasions there was severe though temporary damage to the conjunctiva. The eyes took on a glazed dull appearance, and the corneal reflex was apparently lost. Complete recovery took place in 4-5 days if the animals remained in their cages in a room with a north aspect. When testing the sensitizing effect of unknown materials, untreated control animals were always exposed to the same conditions of light at the same time, and strongly sensitized animals were also included as positive controls.

In experiments made to determine the wave-length of the exciting rays, the radiation from a carbon arc was used and one ear only of the test animal was exposed, the local effect being observed and compared with the behaviour of the unirradiated ear. Sensitization was shown by a local inflammation with swelling and dilation of the capillaries, which lasted for some time after removal from the beam. The intensity of the effect was dependent on the degree of sensitiveness of the animal (dose

of active material given and time after intake), the intensity of the sensitizing rays and time of exposure. In the early days of sensitization, the effects of local irradiation remained *localized* and permitted a very exact estimate of the effect by comparison with the unexposed ear. After feeding the buckwheat flowers for some days, particularly after repeated exposure at short intervals, local irradiation produced a *general* effect with inflammation of the other ear and the nose, tail and paws. Such animals could not be used for further tests with local irradiation.

The effects observed in sensitized mice were similar to those seen in rats, but less distinct. General or local irradiation caused dilation of the veins of the ears but not of the capillaries; the fur of the exposed portions became fragile and fell out and the area became bald, but the hair follicles remained intact and the hair grew again after a few days in the dark. Sometimes this was also seen in rats.

Determination of the wave-length of the activating rays

The following sources of light were used: sunlight during all seasons of the year, both direct and transmitted through ordinary window glass; the unfiltered rays from a mercury-vapour lamp at 52 cm. distance; the rays of an ordinary 1500 W. lamp at 30–38 cm. cooled by a cell 3 cm. deep containing water or a 1% solution of copper sulphate. For local irradiation, the beam of a 10 amp. carbon-arc was focused by quartz lenses and cooled and filtered through a 5 cm. cell of water and generally also through a 5 cm. cell containing copper sulphate solution (1–5%).

Sunshine was in many ways the most convenient of the sources of light, but its irregularity and changing intensity, often from minute to minute, was a drawback, for only those animals could be satisfactorily compared which were exposed simultaneously, since we had no means of recording the intensity. Irradiation could be made much more uniform with the 1500 W. lamp, but here the intensity was inferior, so that the time of exposure had to be greatly prolonged.

For general irradiation with sunlight, the rats were placed in cages composed of thin wire immediately behind open or closed windows with south-east or south-west aspect. When the 1500 W. lamp was used, care was taken to illuminate the whole cage equally; a round tin cage, with solid walls and floor painted white and a lid composed of a lattice made of fine wire was placed below the lamp and filter. The interior was separated into four equal divisions by the same type of netting, so that four animals could be simultaneously and equally illuminated.

For purposes of local irradiation, the rats were lightly bandaged with pads of cotton-wool to a board attached to the optical bench which held the carbon-arc and focusing lenses, so that the irradiated portion of the animal could be fixed in the beam at a measured distance from the burning point. The animals were kept in position by hand, so that one ear only was in the beam of the arc. The intensity of the beam at the spot where the animal's ear was placed was measured before and after the exposure by means of a small photoelectric cell photometer such as is used in photography, its front lens being covered with a diaphragm the size of whose aperture was exactly that of the beam. The values registered were only relative, but were sufficient for our immediate purpose, which was to determine the relative intensity of the rays passing various filters, as compared with those of the unfiltered beam. The filters used were UG 2 and BG 3 of Schott (Jena), a set of Ilford monochromatic spectrum filters (nos. 601-608), by means of which the wave-length of the active rays was determined qualitatively and Wratten filters nos. 15, 21, 29 and 90, for quantitative measurements of relative intensity in addition. The wave-lengths of the rays transmitted by these filters are given in Table 2 and the absorption curves shown in Fig. 1.

RESULTS

Distribution of the activating material in the buckwheat plant

When the rats receiving the diet containing 90% of ground whole buckwheat seeds were exposed to sunshine in front of a window, especially in the months of May and June, they showed a high degree of sensitiveness not shown by controls, and this occurred whether the window was open or closed. They were also sensitive to the beam of the carbon-arc lamp. No sensitiveness to light was observed, however, if the husks were removed from the meal before incorporation in the diet. The details of these experiments and of the following are contained in Table 1.

Examination of the different portions of the growing plant showed that the dried leaves, given in doses up to 0.32 g. daily for 5 weeks, were without effect, but the dried flowers, in daily doses of 0.1-0.2 g., produced highly sensitive animals. With the larger dose, sensitivity to sunshine (September) was present on the third day after the first dose was given and was retained for several weeks after the dose was discontinued. One rat showed erythema of the ears, nose and paws after 5 min. general exposure to sunshine in the beginning of October, and fits occurred after exposure for 55 min.; fits also followed 20 min. exposure to intermittent

TABLE 1. Distribution of light-sensitizing substance in the buckwheat plant.
Tests made with albino rats: those receiving leaves and flowers fed on a diet containing whole wheat or on synthetic complete diet.

Figures in brackets indicate time of exposure in min.

Material	No. of rats	Body wt. at beginning g.	Dose daily, dry wt. g.	Duration of experiment weeks		Source of radiation		Remarks
						Carbon-arc	Sunshine	
Leaves	4	54-80	0.32	3		—	0	—
	9	30-180	0.2	2		+(5)	+(2)	—
Flowers, young	3	40-70	0.1	2		+(6)*	+++(5)	—
	3	116-207	0.05*	2		—	+++(20)	—
	1	167	0.02*	2		—	+++(20)	—
	1	105	0.01*	10		—	+++(60)	—
	3	61-70	0.2	10		—	?_	—
Seeds, including husks	5	38-44	90% in diet	17-24		+(22)	+	—
Seeds, husks removed	3	90-105	92%	10-16		—	++	Sunshine in April little effect; exposure of 5-6 hr. in June severe effect
Maize	4	120-150	90%	3-4		0 (25)	0	—
Wheat	Numerous	—	93%	—		—	0	—

* Per 100 g. rat.

sunshine later in that month. Local irradiation with the beam of the carbon-arc also demonstrated the high degree of sensitiveness of the rats receiving the young buckwheat flowers, far in excess of that shown by those taking the diet composed almost entirely of the seeds. Flowers picked at a later stage, when the fruit was beginning to set, were less active than the younger ones; a daily dose of 0.2 g. sensitized the rats more slowly, and only after the dose had been given for about 10 days. Since the young flowers were found to be the most active portion of the buckwheat plant, all later experiments were made with this material.

Wave-length of the active rays

The search for the active wave-length was narrowed by three facts: (i) sunshine filtered through window glass impermeable to short ultra-violet rays was no less active than the direct sunshine, (ii) the light of an ordinary 1500 W. lamp, rich in visible light and poor in ultra-violet rays, produced all signs of fagopyrism in sensitized animals, (iii) the beam of a mercury-vapour lamp rich in ultra-violet rays and of poor intensity in the visible, at 52 cm. distance, was almost ineffective. Only occasionally did very highly sensitized animals show a slight positive effect, and on the shaved skin of the back this beam produced in highly sensitive animals only the same degree of erythema as was produced in rats that had not received any buckwheat.

The above facts showed that visible rays were most effective in exciting buckwheat disease. This conclusion was confirmed by the result that the carbon-arc beam, which has a high intensity over the whole spectrum from ultra-violet to infra-red, when filtered through filters UG 2 or BG 3 (Schott), which exclude rays of longer wave-length than 405 and 509 $m\mu$ respectively, did not excite any symptoms in highly sensitized animals even after long exposure.

Experiments with Ilford spectrum filters showed that the active rays were only transmitted through nos. 605 yellow-green, 606 yellow, and 607 orange, transmitting respectively the wave-lengths 530-580, 560-610 and 575-690 $m\mu$. This result located the active rays between wave-lengths of 530 and 690 $m\mu$.

Further tests were made with Wratten filters nos. 15 and 21, which cut out rays of shorter wave-length than 510 and 540 $m\mu$, respectively, and with no. 29 which transmits only those longer than 610 $m\mu$. Filters 15, 21 and 29 were found to transmit, respectively, 60, 50 and 10% of the intensity of the original arc beam (see Table 2).

Table 2. Determination of active portion of the spectrum.

Beam from carbon-arc passed through filter + depth of 5 cm. water + 5 cm. 1 % copper sulphate solution; tests made on sensitized rats.*

Numbers in brackets = time of exposure in min.

0 signifies no reaction + signifies definite reaction
 ⊥ signifies slight reaction + + signifies marked reaction

Dose of dried flowers (s = single dose) (d = daily dose)	Filter used	Transmitted wave-lengths mμ	Ratio of intensity of transmitted, to that of original radiation, measured photo-metrically	Reaction of rat to exposure		Ratio of time taken for positive reaction in (a) to that in (b)
				(a) without filter	(b) with filter	
0.05 (d)	UG 2 Schott	281-405	1:2	+ (18)	0 (25)	0
0.05 (d)	BC 3 "	<281-509	1:3	+ + (10)	0 (23)	0
0.5 (s)	601 Ilford	385-475	Not measured	+ + (3)	0 (60)	0
0.5 (s)	602 "	445-495	"	+ + (3)	0 (60)	0
0.5 (s)	603 "	475-520	"	+ + (3)	0 (60)	0
0.5 (s)	604 "	500-545	"	+ + (3)	0 (60)	0
0.5 (s)	605 "	530-580	"	+ + (3)	0 (60)	0
0.5 (s)	606 "	560-610	"	+ + (3)	+ (60)	<1:20
0.5 (s)	607 "	575-600	"	+ + (3)	+ (60)	<1:20
0.5 (s)	608 "	>625	"	+ + (3)	0 (60)	<1:20
0.2 (d)	No. 15* Wratten	>510	3:5	⊥ (1); + (2)	⊥ (4); + (5)	ca. 1:2
0.5 (s)	"	>510	3:5	+ + (2)	+ (2); + + (3-4)	ca. 1:2
0.1 (d)	No. 21* Wratten	>540	1:2	⊥ (0.7); + (1)	⊥ (1); + (2.5)	ca. 1:2
0.2 (d)	"	>540	1:2	+ + (2)	⊥ (2); + (2.5); + + (4)	ca. 1:2
0.5 (s)	"	>540	1:2	+ + (2)	+ + (4)	1:2
0.5 (s)	No. 20* Wratten	>610	ca. 1:10	+ + (2)	0 (60)	0
0.2 (d)	No. 90 Wratten	560-640	ca. 1:8	+ (1)	⊥ (2); + (4)	ca. 1:4
0.5 (s)	"	560-640	ca. 1:8	+ + (3)	⊥ (5); + + (10)	ca. 1:3

Note. Data as to transmission of filters given in column 3 are taken from the following publications: *Jena Coloured Filter Glasses*, Schott, Jena (1902 E); *Ilford Colour Filters*, Ilford, Ltd., London; *Wratten Light Filters*, 15th Edit., Eastman Kodak Co., Rochester, N.Y., 1938.

* Without copper sulphate cell.

The time required to produce erythema of the same sensitized rat when filters 15 or 21 were interposed was increased up to 100%, showing that neither of these two filters held back the active rays. When filter 29 was used, no erythema could be produced, even by long exposure of highly sensitive animals.

Filter 90, which transmitted rays of wave-lengths 560–640 $m\mu$, was permeable only to rays of the critical region, and was found to transmit

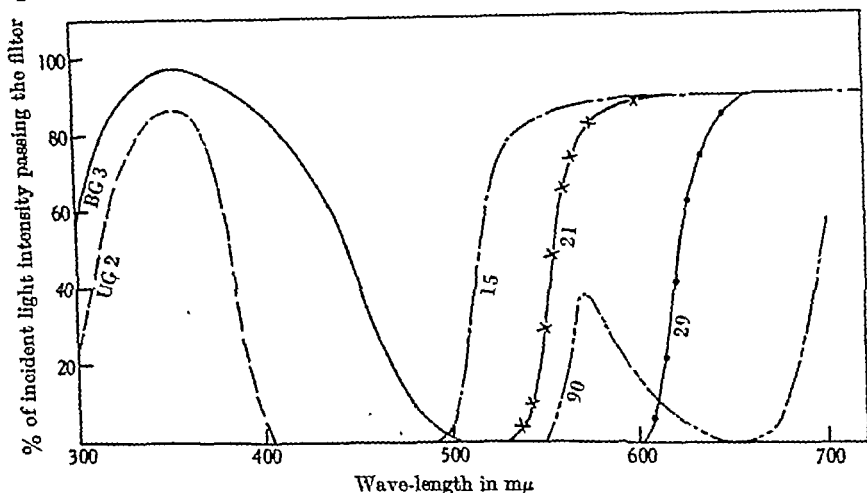


Fig. 1. Transmission curves of the filters used (from the catalogues published by the makers).

only about 12% of the original intensity of the beam. Nevertheless, the biological effect was estimated to be one-quarter to one-third of that of the unfiltered beam.

The results of these tests locate the most active rays in the yellow-orange portion of the visible spectrum between those of wave-length 540 and 610 $m\mu$ (see Table 2).

Minimum activating dose of buckwheat flowers

The minimum sensitizing dose was determined when dried buckwheat flowers were given to rats or mice, either in small daily doses or in one larger single dose (Table 3). In both species a single dose of 0.125 g./100 g. body weight was ineffective, and, in both, 0.25 g. produced slight but distinct sensitivity; with 0.5 and 1.0 g. it was severe. With the last two doses the animals reacted on the day after the dose was given, the reaction was maximal between the fourth and eighth days and disappeared after 14–16 days and 3–4 weeks respectively. Animals receiving the smaller

TABLE 3. Minimum sensitizing dose of dried young buckwheat flowers.

Doses fed to albino rats and mice in one single larger dose or in daily smaller doses.

(a) Given in a single dose					0 = no reaction ⊥ = slight reaction + = definite reaction ++ = marked to severe reaction						
Animal (nos. used in brackets)	Body wt. at beginning of test	Dose (dry wt.) per 100 g. animal	Total amount taken when first signs of sensitiveness observed	Reaction to sunshine through window-glass, 30 min. exposure Days after intake of single dose							
	g.	g.		1	2	3	4-6	7-10	11-14	15-21	Over 21
Rat (2)	35-44	0.125	—	0	0	0	0	0	0	0	0
Mouse (2)	21-23	0.125	—	?	⊥	⊥	+	+	+	+	0
Rat (4)	35-98	0.25	0.25	0	+	+	+	+	+	+	0
Mouse (2)	20-24	0.25	—	+	+	+	+	+	+	+	0
Rat (5)	31-120	0.5	0.5	+	+	+	+	+	+	+	0
Mouse (2)	22-24	0.5	—	+	+	+	+	+	+	+	0
Rat (4)	102-147	1.0	1.0	+	+	+	+	+	+	+	⊥
(b) Given in daily doses											
Sensitiveness first observed			Subsequent reaction to sunshine (S) through window-glass, or to 1500 W. lamp (L) (nos. in brackets indicate exposure in min.)								
Animal (nos. used in brackets)	Body wt. at beginning of test	Dose (dry wt.) per 100 g. animal	Interval after dosing begun days	Total amount taken g.			Days after first positive reaction noted				
	g.	g.					1	2	3		
Rat (4)	40-105	0.01	No positive reaction after 60-90 days				+	+	+	+	+
Rat (4)	85-107	0.02	13-14	0.26-0.28			+	+	(105)	+	+
							+	+	(30)	+	+
							+	+	(120)	+	+
							+	+	(20)	+	+
							+	+	(120)	+	+
Rat (4)	40-133	0.05	5-6	0.25-0.30			+	+	(30)	+	+
							+	+	(120)	+	+
							+	+	(30)	+	+
							+	+	(120)	+	+
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							+	+	(30)	+	+
							+	+	(120)	+	+
	</										

dose of 0.25 g./100 g. weight showed a slight reaction after 24–48 hr., increasing to a maximum after 4 days; they never became as sensitive as those receiving the larger doses and were completely normal after 8–12 days. Different samples of buckwheat gathered in the years 1937–40 varied little in their content of the active material, provided that the preparations were made in the same manner from young flowers separated from the leaves and stems as carefully as possible.

When daily doses were given no sensitiveness was produced, even after 4 weeks, with 0.01 g./100 g. weight; 0.02 g. caused the first signs of sensitiveness after 13–14 days, and this increased in degree until a maximum was reached after 21–24 days. When the daily dose was 0.05 g./100 g. rat, sensitiveness appeared after 5–6 days with the maximum effect 2–3 days later. In both these cases the sensitiveness persisted for several weeks after the doses of buckwheat flowers had been discontinued.

Examination of faeces and urine of sensitized rats for the active principle

The fact that when dried buckwheat flowers were subjected to the action of pepsin or of human gastric juice, only a small proportion of the active material was extracted (see below), suggested that when animals were fed on buckwheat the greater portion of the material might pass through the intestines unabsorbed. To test this point, daily collection was made of the faeces of a large rat, of 200 g. weight, receiving 0.3 g. dried flowers daily, and these were dried and incorporated in the diet of a small one (55 g. weight) for 5 weeks, but no sensitization of the small rat occurred.

Similar experiments were made with the urine of sensitized rats. This was collected from two such rats, of weight 130 and 180 g. each receiving 0.2 g. dried flowers daily, was mixed in the food of one weighing 112 g. and consumed daily for 5 weeks until the rat weighed 179 g.; it remained, however, quite insensitive to light. A second trial gave the same result. In this case a young rat weighing 52 g. received at first the urine from one sensitized rat, and later that from two, 7–12 c.c. daily. This rat also was never sensitive to sunshine.

The urine of sensitive rats was examined for the presence of fluorescent pigments; only in a few instances was a light pink fluorescence observed, and in these the presence of a protoporphyrin could be shown spectroscopically. In no case was there evidence of the presence of red fluorescent pigment.

PART 2. EXTRACTION OF THE ACTIVATING MATERIAL
FROM BUCKWHEAT

Former attempts to obtain an active extract from buckwheat must be considered unsuccessful. Among those who have recorded partial success are Oehmke [1908], Fischer [1909], and Lutz & Schmid [1930]. These three workers used alcoholic extracts obtained respectively from seeds, bran and flowers and gave them, by mouth or injection, to mice or guinea-pigs. The evidence submitted is, however, not conclusive that the symptoms observed were those of buckwheat disease or that a photosensitizing substance was present in the extracts used. Nor have any of the pigments extracted from buckwheat by other investigators, using different methods, proved to be the cause of fagopyrism [e.g. Busck 1905].

In our own experiments attempts were made to extract the active substance with various solvents from finely powdered flowers, whose activity had been previously determined, and, whenever possible, both the extracts and the extracted material were tested on animals.

It was found that when boiling alcohol was used neither the extract nor the extracted material was active, suggesting that the sensitizing substance was heat-labile, and this was confirmed in an experiment in which the flowers were treated with steam. Thereafter all operations were made at room temperature or at 37° C. Extraction was made with several changes of solvent until these had little or no colour. Shaking in a shaking machine was helpful, and often 10-15 extractions were needed, each of 24 hr., with 20 parts of solvent to 1 of the powdered buckwheat.

At first the extracts were evaporated in vacuo at low temperature to a small volume, which was then mixed with rice starch, dried in vacuo and fed thus to the experimental animals, being added to one of the diets described in Part I above. Later, the extracts were concentrated by vacuum distillation at less than 30° C. to about one-tenth of their volume and the concentrate dialysed for some days against running tap water through a cellophane membrane, until the last traces of solvent had been removed. The aqueous dialysates always contained both soluble and insoluble pigments. The latter were filtered off, dried in vacuo and fed separately; the former were concentrated by evaporation in vacuo and then fed.

The solvents used included water, *N*/10 hydrochloric acid, *N*/10 sodium bicarbonate, acid ethyl alcohol (10 parts concentrated hydrochloric acid to 90 parts of 96% alcohol), alcoholic sodium hydroxide (*N*/10 in 80% alcohol), benzene, chloroform, acetone, ether, light

petroleum, glacial acetic acid and trichloroacetic acid 5%. Dioxane, diethylene glycol, glycerol, glycol, and methyl alcohol were also tried in a mixture with 10% glacial acetic acid.

Tests were also made with a series of digestive enzymes: pepsin at pH 2.0, papain at pH 5.0, both directly and after preliminary treatment with *N*/10 HCl, normal human gastric juice and trypsin at pH 8.0 following treatment with pepsin.

RESULTS

Attempts to extract the active pigment with water, glycerol, glacial acetic acid, dioxane with or without acetic acid, benzene, ether, light petroleum, acetone, and chloroform were uniformly negative. No activity was found in the extracts, while the extracted material contained all or most of the original activity.

With a second group of solvents there was more or less destruction of the activity, so that both the extracts and the residue were inactive. This happened when heat or alkali was used, e.g. with steam, boiling ethyl alcohol, sodium bicarbonate solution and digestion with trypsin. Both extract and residue were also negative after extraction with diethylene glycol. The trichloroacetic extract could not be tested as it proved to be poisonous to the rats.

After treatment with pepsin, papain, human gastric juice, and dilute hydrochloric acid, a small proportion of the original activity was found in the extract, while most remained in the extracted material even after six and more extractions. This also happened occasionally with ethyl alcohol, but this solvent usually destroyed all the activity.

The active substance was, however, almost entirely extracted by a mixture of 90% glycol or methanol with 10% acetic acid. Quantitative tests showed that, especially with the latter mixture, the entire sensitizing material was extracted and was present in the water-insoluble portion of the dialysates. This consisted of a mixture of a greyish pink and a brown red powder, the solution of which in methyl alcohol showed a reddish fluorescence. The separation of the pigments is now in progress and will be described by one of us (P. E.) later.

DISCUSSION

Signs of a positive reaction to light in the test animals

In tests for buckwheat sensitivity the most striking and easily reproducible effect was erythema of the naked parts of the skin of the rat, particularly of the ear. This was chosen as the decisive sign of a positive

effect and, when local irradiation was used, comparison of the colour and turgor of the exposed ear with the other one permitted a quick and sure decision, as long as the effect remained localized. As soon as the effect became general, the animals had to be discarded for tests employing local irradiation. The fact that local irradiation produced a local effect in the beginning of the process of light sensitization and a generalized effect later on, throws some light on the mechanism of the light effect on the sensitized, and also perhaps on the normal, animal. It suggests that a certain substance, responsible for the pathological symptoms, might either be formed in, or set free from, the tissues under the influence of light. This question will be investigated further.

It would have been desirable to have a second and perhaps more objective sign of a positive reaction than the erythema. Brockmann, Haschad, Maier & Pohl [1939], in their study of photosensitization by hypericin, mention disturbance of temperature regulation as a typical change occurring after irradiation of animals sensitized to light. In the present work no changes of this kind were observed in highly sensitive animals, after exposure to light, which were in any way different from those seen in unsensitized animals.

The activating wave-lengths

The wave-length of the rays which evoked a reaction in the rats sensitized with buckwheat were found to lie between 540 and 610 $m\mu$. The sun's rays are powerful in this region and well suited to test buckwheat sensitivity, the drawback being the continual change in intensity, and the impossibility of recording it without exact instruments. The radiation from the mercury-vapour lamp has low intensity in rays of the above wave-length; that from the carbon-arc is rich and well suited for tests, but equal and intense illumination can only be obtained over a small area. The gas-filled electric-light bulb also has powerful emission in the critical zone and would be the most suitable for tests if the intensity could be obtained sufficiently great. To avoid heat effects the rays should always be filtered through water or copper sulphate solution.

Details of the physico-chemical properties of the active pigments obtained from buckwheat flowers will be published later, but it may be mentioned here that they showed absorption in the violet and ultra-violet as well as bands between 540 and 610 $m\mu$, and that the fluorescence could only be excited by light within the latter range. It was interesting that the violet and ultra-violet rays which were absorbed by the active substance were without photosensitizing effect on the sensitized animals.

Active portion of the buckwheat plant

The observation that buckwheat seeds contain the sensitizing substance only in the husks and not in the meal, confirms that of Fischer [1909] and of Lutz & Schmid [1930] but is contradictory to that of Oehmke [1908]. He found the meal active as well as the husks, but the details given are too scanty for possible criticism. The leaves were found inactive in our experiments, but the opposite was observed in the careful work of Merian [1915]. We, however, fed only relatively small amounts of the dried leaves; Merian does not say how much he fed. In any case it is certain that the green leaves contain, if any, a very small proportion of the active substance as compared with the flowers or seed husks. The young flower is by far the most active part of the plant, as was noted by several veterinarians [see Merian, 1915], who obtained their evidence from naturally occurring buckwheat poisoning in farm animals. Merian and Lutz & Schmid also found buckwheat flowers highly active, but Brandl and his pupils, Bichlmaier [1912] and Hilz [1913], were unable to produce fagopyrism in various laboratory animals by feeding any portion of the plant; here again, however, the details given are too scanty to permit of any attempt to find an explanation.

Minimum activating dose; absorption and elimination of the active pigments by the test animals

From the pharmacological point of view the photosensitizing pigments of buckwheat are most interesting drugs with a completely cumulative yet reversible effect in the animal.

In tests with single doses of the dried flowers, 0.25 g./100 g. animal was needed to produce light sensitiveness, and about the same total amount was required when the dosing was intermittent. With a daily dose of 0.02 g./100 g. rat, 13-14 doses (or a total of 0.26-0.28 g.), and with a daily dose of 0.05 g./100 g. rat, 5-6 doses (or 0.25-0.30 g.) were needed to provoke the first signs of buckwheat disease. With a daily dose as small as 0.01 g. dried flowers per 100 g. rat, the degree of saturation needed to produce sensitiveness was never reached.

The destruction or elimination of the poison in the animal was very slow, but took place eventually. Sensitivity disappeared 7-32 days after the last administration of a single or intermittent dose, according to its size.

Nature of the sensitizing pigment

The many unsuccessful attempts made by us to extract the active material showed it to be unstable, easily destroyed by heat and alkali and insoluble in organic solvents possessing no hydroxyl group such as benzene, ether, light petroleum, chloroform as well as in water and some organic solvents readily miscible with water, such as glycerol, dioxane, acetic acid, acetone. Inactive pigments with a red fluorescence were, however, extracted from buckwheat flowers both by ethyl alcohol and ether, and use was made of this fact, later, in removing some inactive material by preliminary extraction with ether, before extracting the active substance with the methanol-acetic acid mixture. An acid reaction in general favoured the extraction, as was found in the trials with pepsin and dilute hydrochloric acid.

The results obtained by us with ethyl alcohol were not invariably negative, but never more than a very small proportion of the activity of the original flowers could be found in the extract. Active alcoholic extracts from seeds, husks or flowers have, however, been described by Oehmke [1908], Fischer [1909] and Lutz & Schmid [1930], but their results are inconclusive.

The quantitative tests made with the extracts obtained with mixtures of glycol or methanol with acetic acid showed that with the former most, and with the latter all, of the sensitizing substance was extracted and was found in the water-insoluble portion of the dialysate obtained. The extracted material was quite inactive.

Considering the small proportion of the active substance which could be extracted by peptic digestion or with human gastric juice, it was surprising to find that the faeces of rats receiving daily doses of flowers were free from it. It might have all been absorbed during passage through the intestines, as is suggested by the constancy found in the animal total sensitizing dose; on the other hand, it might have suffered destruction in the more alkaline tracts of the gut.

The urine of highly sensitive rats did not contain the sensitizing agent in recognizable quantity. Smetana [1939] also failed to detect it in the plasma of guinea-pigs fed exclusively on fresh buckwheat plants or seeds. Sheard *et al.* [1928], however, from spectrophotometric studies of the serum of animals sensitized with buckwheat, believed the sensitiveness to be due to the presence in the blood of chlorophyll or of derivatives similar to chlorophyllan (Hoppe-Seyler, 1887).

The occasional increased porphyrin excretion in the urine of highly sensitized rats, recorded in the present paper, has probably no connexion with buckwheat poisoning.

SUMMARY

1. Buckwheat (*Fagopyrum esculentum*) contains in the flower, especially when young, and in the husk of its seed, photosensitizing pigments, which could not be detected in the leaves and stems, in the doses given, or in the buckwheat flour.

2. Feeding of buckwheat flowers or of the husks of the seeds renders partly or wholly unpigmented rats and mice sensitive to light rays of wave-lengths between 540 and 610 $m\mu$.

3. The symptoms of this light disease, fagopyrism, observed in rats and mice were inflammation of the unpigmented portions of the skin particularly on the ears, nose, paws and tail, and of the mucous membranes (conjunctivitis, diarrhoea), and affections of the central nervous system (convulsions).

4. The photosensitizing agent has a well-defined minimum activating dose, viz. about 0.25 g. dried buckwheat flowers per 100 g. animal. The total amount was the same whether given in a series of small daily doses or in one single dose. In the latter case sensitivity to light was present within 24 hr. after the intake and lasted for a considerable time afterwards, this time being in proportion to the size of the dose given.

5. The activating principle could be extracted from dried buckwheat flowers with mixtures of 10 parts of glacial acetic acid with 90 parts of methanol or glycol, and was found present in the water-insoluble portion of the dialysate of the extracts. It was soluble in alcohol with reddish fluorescence. It was easily destroyed by heat or alkali and was extractable to a very small degree, or not at all, by most of the usual solvents.

Our best thanks are due to Sir Charles Martin for the hospitality extended to the Division of Nutrition since the outbreak of war, at Roebuck House, Chesterton, and to him and other friends in Cambridge for giving portions of their gardens for growing buckwheat crops, as well as to the many colleagues and friends who assisted in the tedious work of hand-picking the buckwheat flowers. We also desire to thank Dr Lucy Wills for the supply of human gastric juice needed for some of the tests. One of the authors (P. E.) wishes to acknowledge a Research Fellowship of the Society for the Protection of Science and Learning and a grant for technical assistance from the Ella Sachs Plotz Foundation, as well as the hospitality of the Lister Institute. Our thanks are also due to Miss Else Schweriner and Mr G. Flynn for assistance in the care of the experimental animals.

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FURTHER EVIDENCE CONCERNING THE ROLE OF
THE HYPOTHALAMUS IN THE INDUCTION OF
OVULATION IN THE RABBIT FOLLOWING
INJECTIONS OF COPPER ACETATE

By G. W. HARRIS

From the Anatomy School, Cambridge

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MATING of the oestrous rabbit stimulates a nervous reflex passing via the hypothalamus and pituitary stalk; this in turn causes the liberation from the pituitary of gonadotropic hormone which produces ovulation.

It was found [Fevold, Hisaw & Greep, 1936] that intravenous injections of copper acetate in doses of 10-15 mg. caused ovulation in oestrous rabbits, and the suggestion was put forward that 'the copper salts cause ovulation by increasing the effective activity of the gonad-stimulating hormones present in the blood, and thus the threshold for ovulation is attained'. Evidence that this hypothesis is incorrect has been adduced [Brooks, 1940], since injection of this copper salt intravenously, after section of the pituitary stalk, is ineffective. Probably, then, the copper acetate causes ovulation by stimulation of some part of the nervous pathway to the pituitary.

To test this hypothesis, copper acetate was injected directly into the third ventricle of thirteen oestrous rabbits in doses of 0.05 mg. contained in 0.05 c.c. of water. Ovulation occurred in about 80% of the animals. Control injections were made with normal saline, cerebrospinal fluid, potassium chloride, and calcium chloride solutions. Of sixteen control injections, one rabbit ovulated (saline), four showed haemorrhagic follicles (saline and cerebrospinal fluid), and eleven were negative. It is suggested that the control solutions may have caused stimulation of some nervous pathway in the hypothalamus by virtue of physical effects, such as pressure or temperature changes and that the copper acetate acted in some additional manner due to its chemical constitution. The dose of copper acetate necessary to give ovulation on injection into the region of

the third ventricle is approximately 1/200th-1/300th of the dose necessary to cause the same effect when injected intravenously.

These results support the idea first proposed by Bischoff [1938], that copper salts act on some nervous mechanism. Picrotoxin, metrazol and cadmium salts, which also cause ovulation on intravenous injection, probably act in a similar manner.

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THE RATIO BETWEEN ANTIDIURETIC AND
PRESSOR ACTIVITIES OF POSTERIOR
PITUITARY EXTRACT SUBJECTED
TO MILD HYDROLYSIS

By A. M. FRASER

From the Department of Pharmacology, McGill University, Montreal

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HELLER [1939] has recently studied the effect of hydrogen-ion concentration on the stability of the antidiuretic and pressor activities of posterior pituitary extracts. He found that treating extracts at *pH* values ranging between 0.57 and 10.0 invariably destroyed more pressor than antidiuretic activity. On this work Heller bases his suggestion that the antidiuretic and pressor actions are due to two chemically different principles.

These experiments of Heller are especially interesting because they provide the only noteworthy evidence in the literature that these actions are produced by different substances. Stehle [1934] and others had presented convincing evidence that pressor and antidiuretic actions of pituitary extract are caused by a single principle.

The work presented here, while confirming the results of Heller, supplies reason to believe that this increased ratio of antidiuretic to pressor action in hydrolysed extract is not real, but only apparent.

METHODS

Postlobin-V, Stehle's [1933] preparation of the pressor hormone, was used in this work. The actual extract employed was prepared in this laboratory [Stehle & Fraser, 1935] and contains 200 pressor units and 10 oxytocic units per milligram. Clark & Lubs' [1916] series of buffers was used to obtain the desired *pH* values. The extract was dissolved in the buffer in a concentration of 5 mg./c.c. The solution was sealed in ampoules, heated in a boiling water bath for a given length of time, neutralized to Congo red and again sealed in ampoules and sterilized.

A pressor assay was then carried out using the same preparation of postlobin-V, but untreated, as standard, and finally the quantities, in pressor units, of standard and treated materials, necessary to produce equal antidiuretic responses were determined by techniques described below.

Pressor assay. Chloretonized dogs were employed for this purpose. The standard was dissolved in the same concentration of neutralized buffer as the treated solutions.

Antidiuretic assays. Three methods were employed, viz. the rat method, an intravenous dog method and a subcutaneous dog method.

The rat method of Burn [1931] was used with slight modifications. Food was removed from the cages at 5 p.m. on the evening before the experiment. The following morning, sixteen rats received 2.5% of their body weight of water. Three hours later they received 5% of their body weight of water, following which two groups of four rats each were injected subcutaneously with the standard solution and two groups injected with the unknown solution. The standard solution was adjusted to contain the same concentration of neutralized buffer as the unknown solution. Readings of urine output were made at 15 min. intervals, until considerably more than 50% of the volume of administered water was excreted. Two days later this procedure was repeated, but the two groups which had been given standard solution were now given the unknown preparation and vice versa with the other two groups. The results were now combined, so that the urine output for the whole group of rats was determined for each time interval and for both standard and unknown preparation. By interpolation the time required for the excretion of 50% of the volume of administered water was determined for both standard and unknown. Assays were repeated until one could select a dose of the unknown which gave a 50% end-point in about the same time as a suitable standard dose. (An attempt was made to relate dosage and antidiuretic response in the group of rats employed. The plan was to plot the relation in the form of a curve and thus enable one to determine the strength of an unknown preparation by comparing its antidiuretic response with that of a standard dose. The attempt was unsuccessful because the response to given doses was found to fluctuate considerably from week to week.)

In the intravenous dog method, animals with bladder fistulae were employed. These dogs were trained to lie on a table in which a funnel was placed so that the urine was collected in graduated cylinders as it dropped from the ureteral orifices. The dogs were hydrated by stomach

tubes some hours before, and again, on beginning the experiment. Intravenous injection of the solution to be assayed was made within 5 min. after the diuresis attained a rate of 2 c.c./min. Readings of urine output were made every minute. The time at which the urine flow returned to 2 c.c./min. was taken as the end-point, and time elapsing between the injection and the end-point was regarded as the antidiuretic period. Only one injection was made in each experiment. The antidiuretic period is fairly constant for a given dose of an extract in the same dog. Unknown extracts are assayed by adjusting the doses so that the antidiuretic period matches that of a suitable dose of a known extract.

In the subcutaneous dog method, the animal was prepared as in the intravenous method. On beginning the experiment the administration of water was immediately followed by subcutaneous injection of the extract. The time required for the excretion of 50% of the administered water was taken as the criterion for comparison. For a given dose of extract in the same dog, this time, although somewhat variable, was found constant enough for purposes in this work.

RESULTS

Several samples of postlobin-V were treated at various pH values and for varying lengths of time. In all cases subsequent assay showed less pressor activity than antidiuretic activity, when the latter was measured by the rat method, but these differences were smaller than those observed by Heller.

Two samples were studied in detail and the results are presented below.

Preparation I was treated for 1 hr. at pH 10. A pressor assay showed that about 0.33% of the pressor activity remained. The results of the antidiuretic assays appear in Table 1. The rat assay indicates that its antidiuretic action is about four times that to be expected from its pressor action. However, in the intravenous dog assay the antidiuretic activity of this preparation corresponds closely to its pressor activity.

Preparation II was treated for 45 min. at pH 10. About 1% of its original pressor activity remained. Antidiuretic assays are shown in Table 2. According to the rat method its antidiuretic activity is about three times greater than its pressor activity. But, again, as with Preparation I, the intravenous dog assay indicates approximately equal pressor and antidiuretic action. The subcutaneous dog method, however, although not accurate, yields a result which agrees approximately with that of the rat method.

TABLE 1

In this table the legend 'dose rat antidiuretic units' means the antidiuretic activity as determined by the rat assay, which is four times as great as the pressor assay indicates it should be. P. I=Preparation I; P-V=Postlobin-V.

Rat antidiuretic assay

Name of extract	Exp. 1		Exp. 2	
	P. I	P-V	P. I	P-V
Dose pressor units per 100 g. rat	0.00025	0.001	0.00025	0.001
Minutes to end-point	155	161	163	160

Dog antidiuretic assay (intravenous)

Wt. dog=9.5 kg. Given 250 g. Purina Fox Chow daily at 11 a.m.; 500 c.c. water daily at 5 p.m.; 300 c.c. water daily at 9.30 a.m. Experiment begun at 9.30 a.m.

	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9	Exp. 10	Exp. 11	Exp. 12	Exp. 13	Exp. 14
Name of extract	P. I	P. I	P. I	P-V	P-V	P-V	P. I	P. I	P. I	P-V	P-V	P-V
Dose pressor units	0.01	0.01	0.01	0.01	0.01	0.01	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
Dose rat antidiuretic units	0.04	0.04	0.04	0.01	0.01	0.01	0.01	0.01	0.01	0.0025	0.0025	0.0025
Antidiuretic period in min.	39	39	43	39	40	42	16	14	14	12	10	11

DISCUSSION

On testing the treated preparations by the rat method, one is led to believe, as was Heller, that here is rather direct evidence that the pressor and antidiuretic actions of posterior pituitary extract are due to different substances. However, the subsequent assays by the intravenous dog method seemed to throw doubt on such a conclusion.

The results of the two methods of assay lead to speculation as to the cause of their divergence. Species difference was ruled out as the causative factor by the agreement between the results of the subcutaneous dog method and those of the rat method. Subcutaneous method of administration of the preparations then appeared to be the chief feature necessary for the high antidiuretic value obtained. Strong support is lent to this supposition by the recent work of Noble and his collaborators [Dodds, Noble, Rinderknecht & Williams, 1937; Noble, Rinderknecht & Williams, 1939]. These investigators draw attention to the objections to subcutaneous methods for the antidiuretic assay of impure extracts. They have found that many inorganic and organic substances, when added to posterior pituitary extract, prolong the antidiuresis produced by subcutaneous injection. It seems probable, then, that hydrolytic

TABLE 2

In this table the legend 'dose rat antidiuretic units' means the antidiuretic activity as determined by the rat assay, which is three times as great as the pressor assay indicates it should be. P. II = Preparation II; P. V = Posttobin-V.

Rat antidiuretic assay

Name of extract	Exp. 15		Exp. 16	
	P. II	P. V	P. II	P. V
Dose pressor units per 100 g. rat	0.00033	0.001	0.00033	0.001
Minutes to end-point	175	177	155	101

Dog antidiuretic assay (intravenous)

Wt. dog = 7.0 kg. Given 200 g. Purina Fox Chow daily at 5 p.m.; 250 c.c. water at 9.30 a.m.; 300 c.c. water at 2 p.m. Experiment begun at 2 p.m.

Name of extract	Exp. 17	Exp. 18	Exp. 19	Exp. 20	Exp. 21	Exp. 22	Exp. 23	Exp. 24	Exp. 25
	P. II	P. II	P. II	P. V	P. V	P. V	P. V	P. V	P. II
Dose pressor units	0.0033	0.0033	0.0033	0.0033	0.0033	0.0033	0.01	0.01	0.01
Dose rat antidiuretic units	0.01	0.01	0.01	0.0033	0.0033	0.0033	0.01	0.01	0.03
Antidiuretic period in min.	25	23	24	28	20	29	37	43	43

Dog antidiuretic assay (subcutaneous)

Wt. dog = 9.5 kg. Given 250 g. Purina Fox Chow daily at noon; 250 c.c. water daily at 5 p.m.; 500 c.c. water daily at 9.30 a.m. Experiment begun at 9.30 a.m.

Name of extract	Exp. 27	Exp. 28	Exp. 29	Exp. 30	Exp. 31	Exp. 32	Exp. 33	Exp. 34	Exp. 35	Exp. 36	Exp. 37	Exp. 38	Exp. 39	Exp. 40	Exp. 41
	P. V	P. V	P. V	P. V	P. V	P. V	P. V	P. V	P. V	P. II	P. II	P. II	P. II	P. II	P. II
Dose pressor units	—	—	—	0.02	0.02	0.02	0.06	0.06	0.06	0.02	0.02	0.02	0.02	0.06	0.06
Dose rat antidiuretic units	—	—	—	0.02	0.02	0.02	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.18	0.18
Minutes to end-point	73	73	71	80	90	74	110	93	111	115	94	128	170	151	176
Average time to end-point for equal doses	72	72	72	83	83	74	108	108	112	112	112	128	170	151	176

products contained in these treated preparations likewise prolong the antidiuretic action of the unchanged hormone fraction when subcutaneous injection is employed.

SUMMARY AND CONCLUSIONS

The work of Heller on pituitary extract was repeated and his results in finding the ratio of antidiuretic to pressor activity increased by mild hydrolysis are confirmed. But when the subcutaneous rat method of assaying antidiuretic activity was replaced by an intravenous dog method, hydrolysis was found to have no effect on the ratio between these activities. A subcutaneous dog method yielded results similar to those obtained with the rat method, proving that the cause of the discrepancy in assay results is not a species difference and suggesting that it may be associated with the route of administration. It is concluded that partial inactivation of pituitary extract by hydrolysis produces no real change in the ratio of antidiuretic and pressor activities. One may continue to assume, therefore, that the pressor hormone is responsible for the antidiuretic action of pituitary extract.

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A METHOD FOR ESTIMATING THE FRACTION OF THE VOLUME OF A MUSCLE CONTAINED IN THE VASCULAR SYSTEM

By J. F. DANIELLI (*Beit Memorial Research Fellow*)

From the Department of Physiology and Biochemistry, University College, London, and the Department of Biochemistry, Cambridge

(Received 16 January 1941)

WHEN a muscle is perfused with a solution containing a substance X which can penetrate into the muscle cells, the substance first enters the vascular system, then penetrates into the intercellular spaces and finally penetrates the cell membrane of the muscle fibres. Thus, at least three distinct diffusion spaces are to be recognized: (1) the vascular system, (2) the space between the muscle fibres not included in (1), (3) the intercellular space. The first step in calculating the permeability of the muscle fibres must therefore be to determine what fraction of the amount of X present in the muscle at a given time is present in the vascular system. The concentration of X in the vascular system will, for most substances, be substantially the same as in the perfusion fluid. Hence the amount of X in the vascular system can be calculated if the volume of the vascular system is known.

A convenient procedure for finding the volume of the vascular system is to add a second substance Y (in this paper, haemoglobin) which penetrates the muscle fibres very slowly, if at all. The substance Y will first flood the vascular system and then penetrate into the intercellular spaces. From the time relationships of the increase in the amount of Y , the volume of the vascular system can then be obtained. It is necessary that Y should not cause either dilation or contraction of the vessels, and desirable that it should be easy to estimate in small amounts.

Since the results given here are designed to estimate the accuracy of the method for finding the volume of the vascular system, substance X is omitted and only the non-penetrating substance Y (haemoglobin) studied.

EXPERIMENTAL METHODS

Haemoglobin solutions were prepared by osmotic haemolysis of ox red cells (thrice washed in 1 % NaCl), followed by addition of salts to bring the salt concentration to that of frog's Ringer (6.75 g. NaCl, 0.15 g. KCl, 0.2 g. CaCl_2 , 0.02 g. Na_2HPO_4 per litre). This solution was filtered through a Whatman no. 1 filter paper.

Preparations were made of the hind legs of the frog and these perfused with the haemoglobin solution, through the aorta. Hungarian frogs were used. Frogs of similar size were chosen and where the gastrocnemius muscle weighed less than 1.95 or more than 2.05 g. the experiment was discarded. They were first perfused for 15 min. with 3½ % gum acacia solution, to wash out the erythrocytes already present. The gastrocnemius muscle from one leg was removed and used as a control, and the other leg then perfused with haemoglobin solution for a definite time, after which the gastrocnemius was removed. The muscles were dried by rolling on filter paper and weighed, then roughly minced and extracted twice by stirring for 30 min. with 10 c.c. of distilled water. The extract was centrifuged for 10 min. at 2000 rev./min. and the volume made up to 25 c.c. The haemoglobin was estimated with a photoelectric colorimeter. In the 1938 experiments a special green-sensitive cell was used, and in the 1939 experiments an ordinary cell with a green filter. Extracts of frog muscle, e.g. a gastrocnemius, made after perfusion with gum acacia, contain little haemoglobin, but have a good deal of suspended matter with marked light-scattering powers, which gives an 'apparent haemoglobin' value, as estimated by the photoelectric colorimeter, sometimes nearly as much as one-third of that of a second gastrocnemius muscle (from the same frog) perfused with haemoglobin. There is considerable variation from frog to frog in this respect, but if two gastrocnemius muscles of the same frog are compared, the 'apparent haemoglobin' values do not differ from each other by more than 10 %.¹ Thus, in a muscle perfused with haemoglobin the haemoglobin value given by the photoelectric method is made up of (a) true haemoglobin, and (b) 'apparent haemoglobin'. The amount of the latter is given by the control which was perfused with acacia only. The maximum error in the determination of true haemoglobin, i.e. (true haemoglobin + apparent haemoglobin) in the haemoglobin perfused limb, minus (apparent haemoglobin) obtained from the acacia perfused limb, is not greater than

¹ Seventeen frogs were studied. All except two had muscle pairs agreeing to 5 %; the remaining two lay between 5 and 10 %.

$\frac{30}{70} \times 10\%$, i.e. about 5%. To this must be added a maximum of 1% error due to manipulation and the photocell, giving a 6% maximum error.

Finally, there is an error due to incomplete recovery of haemoglobin, as part remains adsorbed on the minced particles. To correct for this, frog muscle was minced, portions of 2 g. each were weighed out, mixed with known volumes of haemoglobin solution, allowed to stand for 3 hr. and then extracted with water, as in the case of the perfused muscles.

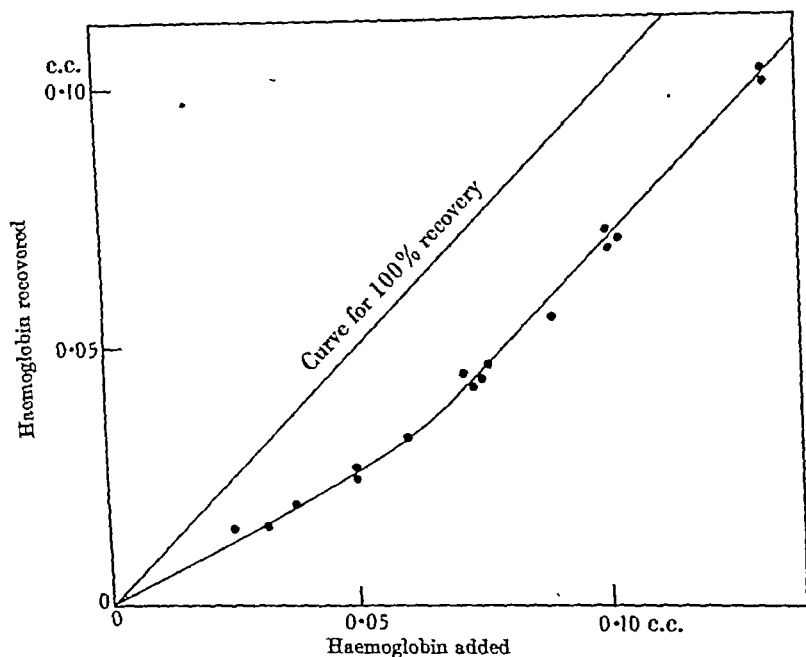


Fig. 1. Results of extraction of mixtures of minced muscle and haemoglobin solution. The figures are in units of c.c. of 7% solution added to 2 g. of muscle.

The amount of haemoglobin recovered was plotted against the amount added (Fig. 1); this curve was then used to correct the values for perfused muscles. The possibility that this correction is inadequate will be discussed later. The uncertainty in the value of this correction involves a further error of $2\frac{1}{2}\%$ in a determination of 0.1 c.c. of haemoglobin solution, giving a maximum possible error for a single determination of $8\frac{1}{2}\%$.

Chloride determinations were also made on the muscle extract, using the method of Sendroy [1937].

From the relationships between the volumes of undissolved material and extractant (2–2.5 c.c. of solid, twice extracted with 10 c.c.) it was

concluded that between 4 and 6 % of the chloride was not extracted. This was checked in some cases by grinding with sand the residue left after extraction, and extracting again with three 10 c.c. portions of distilled water (see Table 1). The amount of chloride obtained by the

TABLE 1

Exp. no.	% of chloride removed by first two extractions	% of chloride removed by three further extractions
7	95.4	4.6
8	96.1	3.9
15	94.9	5.1

first two extractions was therefore taken as 95 % of the chloride actually in the muscle. The chloride determinations were made in duplicate and never differed by more than 2 %; the corrected values are therefore not subject to an error of more than ± 3 % for a given muscle.

RESULTS

Fig. 2 shows the results obtained with 'autumn' Hungarian frogs in 1938 and 1939, expressed as percentage of the total volume of the muscle occupied by haemoglobin solution. It was assumed that the haemoglobin solution in the vascular system is of the same concentration as the perfusion fluid. Each point on the curve represents results obtained by perfusion of a single frog. The haemoglobin volume rises to 5 % in 15–20 min., when perfused under a pressure of 15 cm. of water. Thereafter it remains almost constant, perhaps rising slightly, for up to 5 hr. The average volume of the vascular system of the gastrocnemius is obtained by extrapolating the linear portion of the curve to a time lying between $7\frac{1}{2}$ and 10 min.—a volume in this case not significantly different from 5 %.

The chloride space is expressed as percentage of the total volume of the muscle which would be occupied by chloride solution if, in the muscle, it were present in all parts to which it has access in the same concentration as in frog's Ringer solution. This is the convention used by Fenn and Eggleton [see Fenn, 1936]. The average chloride space of the controls, i.e. of muscles perfused for 15 min. with $3\frac{1}{2}$ % acacia solution, was 13.9 %. On changing to 7 % haemoglobin the volume of the chloride space fell sharply, settling to a value of about 10.5 %. This drop is due to the fact that 7 % haemoglobin solution has a much higher colloid osmotic pressure than has 3.5 % acacia solution, so that, on changing from acacia to haemoglobin solution, fluid is withdrawn from the inter-cellular spaces into the capillaries. This loss of fluid is also shown by the

drop in weight of the perfused preparation which is observed on changing from 3½% gum acacia to 7% haemoglobin. Roughly, half of the chloride space, under these conditions, is actually part of the vascular system.

For the purpose of attaining greater accuracy, it was decided to use as high a concentration of haemoglobin as possible. On the other hand, it was not desired to expose the preparations to these solutions for longer than necessary, although later experiments have shown that the haemoglobin solutions are not harmful.

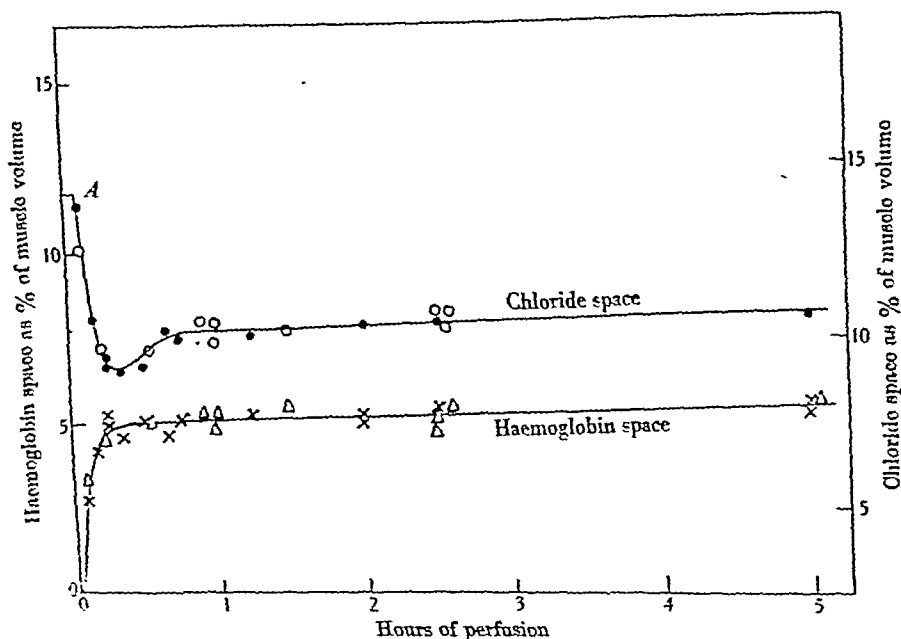


Fig. 2. The variation with time in haemoglobin and chloride space of perfused frog gastrocnemius muscle. Chloride space: ●, 1938; ○, 1939; haemoglobin space: x, 1938; Δ, 1939. Point A is the average chloride space of the control muscles from the same frogs not perfused with haemoglobin.

A further series of experiments was made in which the initial perfusion with acacia solution was continued for 45 instead of 15 min. This made no significant difference to the volume of the vascular system.

DISCUSSION

Krogh [1922] made an estimate of the fraction of muscle volume occupied by capillaries by counting the total number of capillaries in a muscle, and assuming their diameter equal to that of a frog erythrocyte—15μ: this gives 7.1% of the muscle volume as capillary space. Later

he found that in resting frog sartorius muscle the average diameter was 4.3μ , and in stimulated muscle 7μ .¹ In the perfusion experiments described here the capillaries were probably mostly dilated (though not so much as in the presence of capillary poisons), so that 7μ may be taken as a rough value for the minimum capillary diameter. This gives 1.8 % as the minimum capillary volume in the sartorius muscle. If these figures are also applicable to the gastrocnemius (which may well be doubted) they suggest a rough value for the minimum capillary volume of about 2 %. This value does not contradict the value of 5 % obtained here, which includes the volume of arteries, arterioles, veins and venules. By another method, to be published later, a value of 5.2 ± 0.5 % has been obtained for the volume of the vascular system, in good agreement with that obtained here.

The results of Fig. 1 suggest that haemoglobin does not penetrate into the intercellular spaces. This, however, is not the case. On beginning a perfusion with haemoglobin the muscles flush a delicate pink in the first 20 min. and thereafter, during the next 5 hr., become deeply stained. The secondary slower deepening of colour must be due to haemoglobin penetrating the capillary walls. Microscopic observation of the surface of a stained muscle shows that the red colour (unlike carbon particles) is not confined to the capillaries, and the oedema fluid collecting between the muscles and the skin contains much haemoglobin. But it is impossible to remove this intercellular haemoglobin by two extractions of coarsely minced muscle with distilled water.

It has been mentioned above that part of the haemoglobin in the capillaries is adsorbed on the minced tissue, and a correction was made for this by extracting mixtures of minced muscle and haemoglobin solution. As extraction with water does not remove the intercellular haemoglobin, it may be doubted that the extraction of simple haemoglobin-minced muscle mixtures is a sufficient control on complete extraction from the interior of the vascular system. Two comments may be made on this: (1) by another method given in the second paper in this series, using galactose and maltose instead of haemoglobin, a value of 5.2 ± 0.5 % has been obtained, compared with 5 % for the haemoglobin method. This suggests that the method cannot be greatly in error; (2) coarse mincing does not break down the structure of a muscle, as is shown by microscopic observation of fragments of muscle perfused with a solution containing carbon particles; consequently the alternate compression and expansion due to stirring in extraction should secure

¹ The erythrocytes are greatly distorted during passage through these narrow capillaries.

efficient flushing of the vascular system but not necessarily of the intercellular spaces. Substances of high molecular weight removed from the intercellular spaces possibly must first diffuse into the capillaries—a very slow process in the case of large molecules such as haemoglobin. It is therefore reasonable to expect that the intercellular haemoglobin should be more difficult to extract than that present in the vascular system.

How far the chloride over and above that in the vascular system represents intercellular fluid is a matter of dispute [see e.g. Fenn, 1936; Eggleton, Eggleton & Hamilton, 1937; Conway & Kane, 1934]. But even if the whole of the chloride were intercellular, in the experiments reported here the volume of the intercellular space would be only 5 % of the muscle volume, in the case of a muscle after perfusion for an hour or more with haemoglobin solution, and about 9 % just prior to the entry of the haemoglobin solution. The volume of this intercellular, non-vascular, space is determined by the hydrostatic pressure in the vascular system, by the colloid osmotic pressures in the vascular system and in the intercellular space, by the rate of removal of fluid by the lymphatic system and by the volume and packing of the muscle fibres. It is therefore very susceptible to variation in experimental conditions.

SUMMARY

1. A method is given for determining the volume of the vascular system of a perfused muscle, based on determination of a substance in the perfusion fluid.
2. Using haemoglobin as the determined substance, the volume of the vascular system of the frog gastrocnemius muscle is found to be 5 % of the muscle volume.

I am indebted to Dr D.M. Needham and Prof. Sir F.G. Hopkins for reading the manuscript, and to the Government Grants Committee of the Royal Society for a grant covering part of the expenses.

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THE VOLUME OF THE VASCULAR SYSTEM, AND PENETRATION OF SUGARS FROM THE VASCULAR SYSTEM INTO THE INTERCELLULAR SPACE

By J. F. DANIELLI (*Beit Memorial Research Fellow*)
AND H. DAVSON

*From the Department of Biochemistry, Cambridge, and the Department
of Physiology and Biochemistry, University College, London*

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IN a previous paper [Danielli, 1941] a method was given of measuring the volume of the vascular system of a perfused muscle, and results given for perfusion with haemoglobin solution. In this paper, results are given for the penetration of sugars into the intercellular non-vascular spaces of frog muscles, when perfused with sugars dissolved in haemoglobin-Ringer's solution. The method used was to measure the total extra sugar present in a muscle after perfusion and subtract from this the amount present in the vascular system. The remainder must have penetrated into the non-vascular spaces of the muscle.

EXPERIMENTAL

The composition of the Ringer solution was 6.75 g. NaCl, 0.15 g. KCl, 0.2 g. CaCl_2 , 0.02 g. Na_2HPO_4 , 70 g. haemoglobin per litre. The whole of the haemoglobin used in the actual experiments reported here was prepared in one batch. Ox red cells were washed four times with 1 % NaCl solution in the centrifuge, haemolysed with distilled water, salts were added to bring the composition up to that of frog's Ringer, and the particulate detritus was centrifuged off as far as possible. The solution was diluted with frog's Ringer until it contained 8 % of protein by weight. This solution was stored, frozen at -10°C ., in one litre samples. When thawed and oxygenated by a stream of oxygen bubbles the solution had a pH of 7.4. Preparation of this large batch was necessary, because preliminary experiments had shown that, whereas the results obtained with any individual batch of haemoglobin were consistent, results with

different batches differed considerably in the average rate of penetration of sugar. This is probably due to particulate matter not removed by centrifuging. When required for use the haemoglobin was brought down to 6 % by addition of either Ringer's solution or Ringer's solution + sugar.

Hungarian frogs were used, which had been kept over the winter, and were used between April and August. Preparations of the hind legs were perfused through the aorta for 30 min. at a pressure of 15 cm. of solution; then the gastrocnemius and sartorius muscles of one leg were removed, dried by rolling on filter paper, weighed and frozen solid with solid CO_2 . The perfusion of the other leg was continued with haemoglobin-Ringer in which 10 % of the salts were replaced by an osmotic equivalent of sugar. After an interval varying between 10 min. and $3\frac{1}{2}$ hr., the muscles of this leg were removed, dried, weighed and frozen.

The frozen muscles were crushed by pounding in a steel cylinder (previously cooled with solid CO_2) and ground into small fragments, using a pestle and mortar, together with some solid CO_2 . A definite volume (3 c.c. for a gastrocnemius, 1 c.c. for a sartorius) of a solution containing $0.5\text{ M H}_2\text{SO}_4 + 0.5\text{ M ZnSO}_4$ was poured into the mortar. The whole mass froze solid, and was allowed to thaw with constant stirring. When thawing was complete an equivalent volume of NaOH solution was added. The concentration of NaOH was adjusted so that, when added to an equal volume of the $\text{H}_2\text{SO}_4 + \text{ZnSO}_4$ solution, the final solution was just pink to phenolphthalein. The precipitate so formed was centrifuged down and washed 3 times with distilled water. The volume of the combined supernatant fluid was made up to 10 c.c. (for a sartorius) or to 50 c.c. (for a gastrocnemius). Duplicate sugar and Cl^- determinations were made on these solutions.

About 1 % of the sugar and chloride was left in the precipitate. This source of error was neglected in the results given below.

RESULTS

(a) Controls and estimation of errors

Before beginning the perfusion with a solution containing sugar, the muscles were perfused for 30 min. with haemoglobin-Ringer containing no sugar, to bring the muscles of both legs into a standard state. The sugar values and chloride values for the corresponding muscles of the two limbs are then closely similar and remain similar on further perfusion. Table 1 shows two sets of sugar determinations, one after perfusion for 50 min., the second after perfusion for 150 min.; the difference

TABLE 1. Muscle sets *A* and *B* perfused for the same length of time, with sugar-free haemoglobin-Ringer's solution. Sugar values as c.c. of maltose solution

Perfusion time min.		<i>A</i>	<i>B</i>
50	Gastrocnemius	0.351	0.344
	Sartorius	0.089	0.093
150	Gastrocnemius	0.258	0.260
	Sartorius	0.057	0.061

between the muscles *A* and *B* is partly due to errors in manipulation and in the sugar determination, but may in part be due to real differences in the amounts of the substances in the muscles which are estimated as sugar. The highest variation found for a pair of gastrocnemii was 3 %, and of sartorii 7 %, when comparing averages of duplicate determinations. These values, then, will be the maximum errors due to the control muscles. Then, on changing to the sugar-containing solution, one limb only is perfused, and the increase of the sugar value of this limb, compared with that of the limb perfused for the first 30 min. only, is taken as equivalent to the extra sugar present as a result of perfusing with sugar solution. It is, however, possible that in addition to sugar entering the muscle from the sugar solution, some of the 'apparent' sugar still present after the first 30 min. may be washed out by the perfusion fluid. The magnitude of this effect can be studied by perfusing with sugar-free solution, removing the muscles from one leg *A*, after 30 min., and the other, *B*, after $(30+x)$ min. Table 2 shows two typical sets of results

TABLE 2. Muscles perfused with haemoglobin-Ringer's solution without sugar. Set *A* removed after 30 min., set *B* after $(30+x)$ min. Sugar values as c.c. maltose solution

<i>x</i>		<i>A</i>	<i>B</i>
20	Gastrocnemius	0.103	0.101
	Sartorius	0.034	0.031
120	Gastrocnemius	0.127	0.121
	Sartorius	0.036	0.038

with $x=20$ and 120 respectively. In eleven such experiments with $x=120$, sartorius *B* was less than sartorius *A* by an average of 6 %, and gastrocnemius *B* was less than gastrocnemius *A* by an average of 4 %. Individual variations were sometimes larger, the largest change found for a gastrocnemius being 6 %, and for a sartorius 10 %. In eight experiments with $x=20$, the maximum differences were 3.5 % for the gastrocnemius and 6 % for the sartorius. The total of substances estimated as sugar in the resting muscle does not therefore vary very markedly with perfusion prolonged up to $2\frac{1}{2}$ hr.

If, after perfusion with sugar-free solution for 30 min., perfusion is continued with solution containing sugar, the sugar value rises by about 100 % for the sartorius, and rather more than 50 % for the gastrocnemius, in the first 15 min., and thereafter continues to increase. With this information we can now estimate the maximum error to be expected in a single determination. In the case of the gastrocnemius, the maximum error due to the control muscle (perfused for 30 min. with sugar-free solution) has been given above as 3 % of the resting value, i.e. 6 % of the increase after 20 min. perfusion with sugar solution. Then the sugar value obtained after perfusion for 20 min. with sugar solution may be in error by 3.5 % of the value of the control value, i.e. 7 % of the increase. Hence, if all the errors act in the same direction, a single determination may be in error by ± 13 %. For the sartorius the value is ± 13 % also. The maximum scatter of the results actually obtained after any given period of perfusion with sugar was gastrocnemius ± 12.5 % and sartorius ± 9 %. Most of the results were grouped more closely than this suggests, probably because the maximum possible errors are compounded of many individual errors, which will often tend to cancel out.

For perfusion for longer times with sugar solution the amount of sugar in the muscles increases, so that there is less error in the actual amount of sugar determined; on the other hand, the error due to the blank tends to become larger, so that the total error remains roughly constant.

(b) *Variation in the 'sugar space' and 'chloride space'
with time of perfusion*

Fig. 1 shows the chloride and sugar 'space' for gastrocnemius muscles perfused with solutions in which 10 % of the salts were replaced with an osmotic equivalent of galactose or maltose. In calculating the 'spaces' it has been assumed that chloride and sugar, where present in the muscle, are present in the same concentration as in the perfusion fluid. This assumption is, of course, not true but it is a useful convention for presenting results.

The sugar 'space' increases rapidly in the first 10–20 min. up to a value of 6 %. After this there is a slower linear increase lasting for at least 3 hr. At the end of this time the sugar 'space' is about 14 %, roughly equal to the initial chloride space. If we extrapolate the linear portion of the sugar curve to a time 10 min. after starting perfusion with sugar solution, a value of 5.2 ± 0.5 % is obtained. One would expect this value to be close to the volume of the vascular system, since the vascular system must be the first part of the muscle to become filled

with sugar solution. It is in reasonable agreement with the value of 5 % obtained previously for the volume of the vascular system, based on haemoglobin estimations [Danielli, 1941]. We shall therefore assume that the sugar values in excess of 5.2 % represent sugar which has penetrated across the capillary membranes into the non-vascular inter-cellular space. This contention is supported by the fact that the average

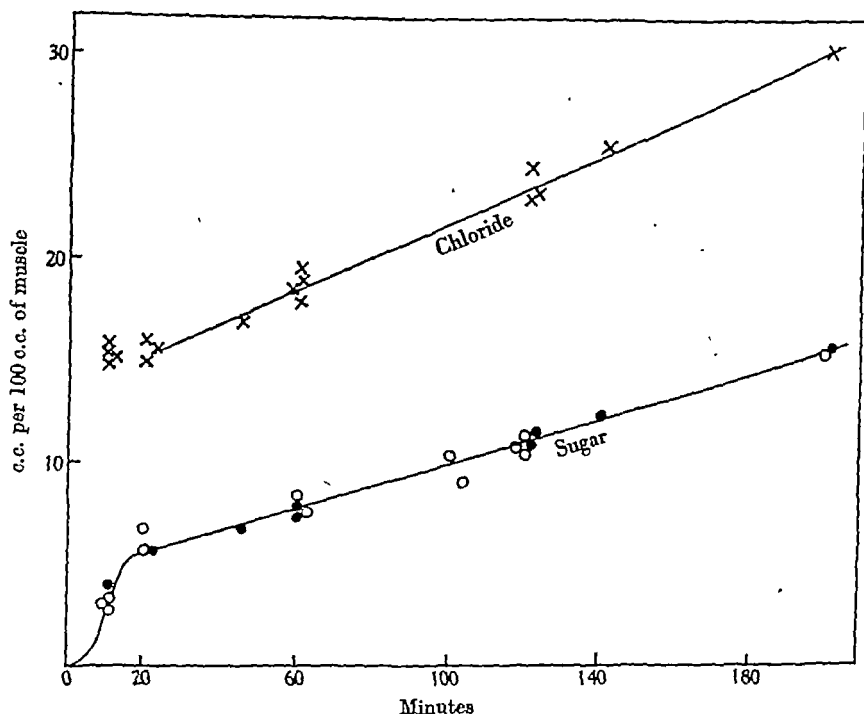


Fig. 1. Variation with time in the sugar and chloride spaces of perfused frog gastrocnemius muscle. Chloride space, x. Sugar space: maltose, ●; galactose, ○.

volume of the vascular system at 120 min., as given by haemoglobin determinations on five frogs perfused with haemoglobin-sugar-Ringer, was 5.1 %.

The linear increase in the amount of sugar in the muscle after the vascular space is flooded is typical of perfusion experiments, and quite different to what is found when a dissected muscle is soaked in sugar solution, when a curve is obtained with a slope continually diminishing with time. The reason for this difference is that in the case of a soaked muscle, the rate of entry of sugar is $ds/dt = -k(C_0 - C_t)$, where C_0 is the external concentration of sugar and C_t the concentration in the muscle.

Hence as C_i increases, ds/dt falls off. But in the case of a perfused muscle, penetration from the vascular system into the intercellular spaces is not a simple diffusion process, but is to a large extent controlled by the bulk flow of fluid from the capillary into the intercellular spaces, the controlling force being the difference between the hydrostatic pressure in the capillary and the colloid osmotic pressure difference across the capillary wall [Starling, 1895]. In the case of the frog, the excess of fluid filtered off in this way is normally removed by the lymphatic system: in these perfused preparations the excess collects in the large spaces between the muscles and the skin. Thus, there is a constant steady flow of fluid from the muscles to the subcutaneous spaces and, although the weight of individual muscles varies but little, the weight of the preparation as a whole increases considerably (about 10 % in 3 hr.). It is this constant flow which is responsible for the steady increase in the muscle sugar, at least during the first hour of perfusion. Later, as the intercellular spaces become full (after the third hour?) there should be no further increase in muscle sugar, except by penetration into the fibres: discussion of this is reserved for a later paper.

The gastrocnemius chloride space (Fig. 1) appears to remain steady for about 20 min., then increases linearly from 14 up to 30 % at 200 min. These figures are slightly misleading for the earlier times. The chloride space at zero time is calculated on the basis of 100 % Ringer solution. For later times, since the perfusion fluid is changed to 90 % Ringer (to keep the osmotic pressure constant in spite of addition of sugar), the chloride space is calculated on a basis of 90 % Ringer. Thus there is an instantaneous rise in the calculated Cl space at zero time, which is entirely artificial in character. The true chloride space does not change at all in the first few minutes. In a few experiments (not on Fig. 1), where the muscles were perfused with sugar solution for 5 min. only, this 'rise' was actually 'observed'. Opposing this artificial rise is a real tendency for the chloride space to diminish immediately the sugar-containing solution reaches the capillaries, due to the tendency of chloride to diffuse from the intercellular spaces into the capillaries, which have temporarily a lower chloride content, per c.c. of fluid.

Thus the apparent constancy of the chloride space during the first 20 min. is probably due to a balance between these two opposing factors. The reason for the linear increase in chloride space after 20 min. is at present unknown.

Results for the sartorius muscle, shown on Fig. 2, closely parallel those for the gastrocnemius. The linear portion of the sugar curve, exter-

polated to 10 min., gives a value of 10.5 ± 0.5 % for the volume of the vascular system. After 200 min. perfusion, the sugar space increases to 27 %, roughly equal to the initial chloride space. The chloride space is 27 % at zero time and increases to 45 % after 200 min. perfusion.

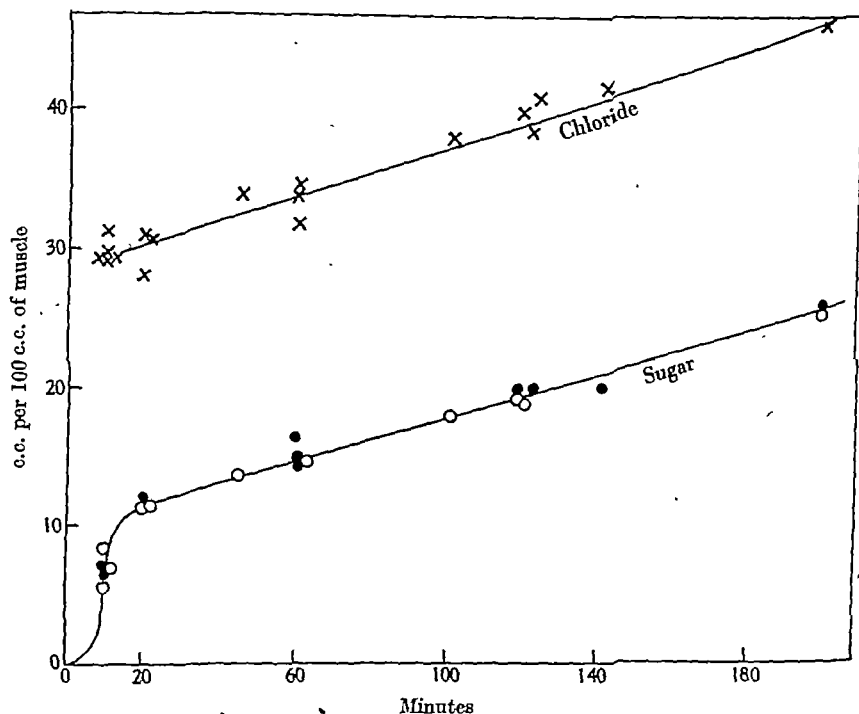


Fig. 2. Variation with time in the sugar and chloride spaces of perfused frog sartorius muscle. Chloride space, x. Sugar space: maltose, ●; galactose, ○.

DISCUSSION

It is assumed that, except at the pores, the capillary wall is practically impermeable to sugars and that through the pores there is a mass flow of fluid, as suggested by Starling, carrying all the non-colloid, non-ionic constituents of the perfusion fluid across the capillary wall in the same proportion as they exist in the perfusion fluid itself. This is supported, in this particular case, by the fact that the slopes of the curves for maltose and galactose penetration are the same; this is what would be expected on the Starling filtration hypothesis, whereas if penetration occurred by simple diffusion across the capillary wall, the rates of

penetration should be as 1.4:1. These results may, therefore, be regarded as offering additional support to Starling's hypothesis.

In both the gastrocnemius muscle and the sartorius muscle, sugar penetrates from the capillaries into the intercellular spaces at a rate which is linear for at least 3 hr. After this time the sugar 'space' is equal to the initial chloride 'space'. The rate of increase of muscle sugar over this period is a measure of the capillary permeability. The sugar space after 3 hr. perfusion is of the same magnitude as that found by M.G. Eggleton [1935] with soaked muscles.

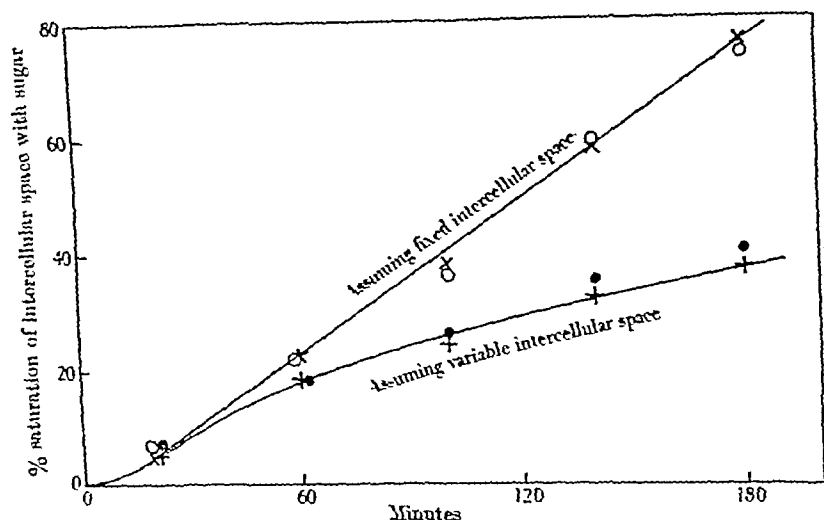


Fig. 3. Degree of 'saturation' of the non-vascular intercellular space with sugar after various times of perfusion. Gastrocnemius, $\times +$; sartorius, $\bigcirc \bullet$. The curves were drawn for the gastrocnemius.

The chloride 'space' rises above its initial value as sugar penetrates into the intercellular space. Whether this rise is due to an increase in the intercellular space is not clear. If the intercellular space does increase, the volume of the muscle fibres must diminish, since there is little change in the total weight of the perfused muscles.

Fig. 3 shows the increase with time in the percentage saturation with sugar (considering the perfusion fluid as 100 %) of the non-vascular intercellular space, assuming that no sugar penetrates into the fibres. The curves were drawn for the gastrocnemius muscle, but also fit the points for the sartorius rather closely. The upper line is for values based on the assumption that the non-vascular intercellular space is equal to

the initial chloride space, less the vascular space. On this assumption the volume of the non-vascular space does not vary with time. The lower line is for values based on the assumption that the non-vascular intercellular space is equal to the chloride space less the vascular space. On this assumption, since the chloride space varies with time, so does the non-vascular intercellular space. It is clear from Fig. 3 that the two assumptions lead to quite different values for the concentration gradient across the cell membrane. A similar variation in chloride space occurs when frogs are perfused with solutions containing polyhydric alcohols. These alcohols penetrate into the fibres at speeds which diminish as the number of OH groups is increased [Danielli, unpublished]. Obviously in the calculation of permeability constants for a molecule such as glycerol, it is essential to know which of the two alternative assumptions given above for the volume of the non-vascular intercellular space is correct.

It is curious that, although the gastrocnemius and sartorius muscles have values for the vascular, non-vascular intercellular, and chloride spaces which are quite different for the two muscles, the degree of saturation of the non-vascular intercellular space is the same in the two cases at any given time, as is shown by Fig. 3. This coincidence means that, although the physical proportions of the two muscles are quite different, yet any nutritious substance present in the vascular system is equally available to the *fibres* of both muscles. This may be of importance in the intact animal.

SUMMARY

1. When a muscle is perfused with haemoglobin-Ringer solution containing sugar, both the chloride 'space' and the sugar 'space' increase with time. After the first 20 min. the increase is linear in both cases. The volume of the sugar 'space' is always less than that of the chloride 'space'.

2. The volume of the vascular system, as determined from the sugar space-time curve, is 5.2 ± 0.5 % of the muscle volume for the gastrocnemius, and 10.5 ± 0.5 % for the sartorius.

3. Although the volumes of the vascular and non-vascular intercellular spaces are different in the two muscles, the degree of saturation of the intercellular space with sugar is the same for the two muscles for any period of perfusion up to 3 hr. It is suggested that this may be of significance for the nutrition of the fibres in the intact animal.

We are indebted to the Director of the Low Temperature Research Station for cold storage facilities, and one of us (J.F.D.) is indebted to the Government Grants Committee of the Royal Society for a grant partly defraying the expenses. We are indebted to Dr Kerly for advice in the early stages, and to Dr D. M. Needham for reading the manuscript.

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AN INVESTIGATION OF SIMPLE METHODS
FOR DIAGNOSING VITAMIN A DEFICIENCY
BY MEASUREMENTS OF DARK ADAPTATION

BY D. J. DOW AND D. M. STEVEN

From the Department of Biochemistry, Oxford

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MEASUREMENTS of the speed and amount of dark adaptation have been used for a number of years to estimate the level of vitamin A nutrition of persons, and to detect states of deficiency. All such tests are based on the fact that vitamin A is commonly the critical factor in the cure of nutritional night-blindness in man and animals [Fridericia & Holm, 1925; Tansley, 1931; Wald, Jeghers & Arminio, 1938; Wald & Steven, 1939; Hecht & Mandelbaum, 1939, 1940], and also on the part played by it in the photochemical cycle of visual purple in the rod cells of the retina [Wald, 1935]. Night-blindness is characteristically a defect of scotopic, or rod, vision, and precise information about the photochemical processes of the retina, in which vitamin A is involved, is at present available for the rods only.

Many of the methods that have been devised in recent years for the clinical diagnosis of vitamin A deficiency are inadequate, since certain precautions that are necessary in the present state of knowledge of visual processes have been ignored. The aim of many of these clinical tests has been to obtain convincing results rapidly, and therefore the measurements of visual threshold have been made during the early stages of dark adaptation, following light adaptation for various periods to various brightnesses of illumination. A method that has commonly been used, which may be referred to as the 'recovery time' type of test, is to measure the time in seconds that the subject requires in total darkness in order to perceive a test field of constant brightness, following a short period of light adaptation [Pett, 1939; Goss, Farmer & McFarlane, 1941; Steele, 1940; Haines, 1938; Gridgeman & Wilkinson, 1938; Jeans & Zentmire, 1934]. An abnormally long recovery time is taken to

indicate a state of vitamin A deficiency, and if the result of therapy is to shorten the time appreciably, the diagnosis is said to be confirmed.

In tests of this type the particular part of the retina stimulated is rarely held constant in any way, so that any given reading may be obtained by stimulation of any part of the retina. As the rod-cone composition of the retina varies greatly throughout its sensitive area, cones only being present at the fovea and almost exclusively rods at the periphery, and as the dark adaptation characteristics of rods and cones differ in important respects, it is important in all measurements to stimulate a defined area, in which the proportions of the two types of sensitive elements is known at least approximately. Measurements of the early stages of dark adaptation, following even a short period of light adaptation, mainly record the initial adaptation of the cones, and the relation of vitamin A to the photosensitive pigment of the cones is still unknown. The most sensitive single index available of the state of vitamin A nutrition is the final threshold of the rods [Hecht & Mandelbaum, 1939, 1940; Wald *et al.* 1938; Steven & Wald, 1941], to obtain which requires at least 30 min. of dark adaptation, and which can be measured accurately by the adaptometer method. Although cone adaptation seems also to be affected during states of vitamin A deficiency, the derangement is usually less than that of the rods, and the photochemical basis remains unknown.

The following tests were therefore made in order to investigate consequently the relation between 'recovery times' and the final threshold of rod vision.

EXPERIMENTAL

The dark adaptation characteristics of two well-nourished subjects in good health were determined with an adaptometer. The method used was that described by Steven & Wald [1941] and Wald [1941]. Readings of the visual threshold of each subject were made on a circular retinal field, $2^{\circ} 27'$ in diameter, 6° above the fovea. This area was exposed to flashes of $\frac{1}{50}$ sec. duration. The colour of the test flash was white and readings were binocular. The complete course of dark adaptation was recorded for 40 min. in darkness, following an initial period of light adaptation for 3 min. to a screen of a brightness of approximately 2000 millilamberts. The readings of visual threshold were recorded in micro-millilamberts (10^{-9} lamberts), and were plotted on logarithmic scale.

The adaptometer, which has been described in detail by Wald [1941], consists of a box containing batteries and a control panel, and a test unit. The latter is built from a section

of brass tubing about 4 in. long and 2 in. in diameter. Mounted in the base is the test lamp, which is on a potentiometer circuit, and which is calibrated photometrically. This illuminates the test field of opal glass, which is at the other end of the tube, for flashes of $\frac{1}{8}$ sec. duration, through a camera shutter mounted in the central portion. The fixation lamp is on a separate circuit, and is mounted in a small housing on top of the test unit. It shines through a pinhole aperture, over which is a deep red filter (Wratten no. 70). Attached in front of the test unit is a funnel-shaped mask, which holds the head of the subject in the correct position, 12 in. from the test field. The relative positions of the subject's eyes, the test field and the fixation point are such that the test field subtends an angle on the retina of $2^{\circ} 27'$, 6° above the fixation point. The whole test unit is mounted on an adjustable camera tripod.

The test unit is connected with the box containing the batteries and control panel by a length of light, insulated wire. On the control panel are switches for the test and fixation lamp circuits, a resistance attached to a scale for varying the brightness of the test lamp, and a second resistance and voltmeter for setting the line voltage of the test circuit. To make a reading of the visual threshold the subject is seated comfortably with his face against the mask and his eyes focused on the red fixation point. The operator illuminates the test field by clicking the camera shutter. Starting with the brightness of the test lamp at a subthreshold level, he increases it by a small regular amount between each exposure until the subject reports seeing it. The time and scale reading of brightness are then noted. The increase in brightness of the test field between successive exposures is insignificant, and a high degree of accuracy is therefore obtained in each measurement of the visual threshold. After a few trial readings the great majority of subjects are found to give data of a high degree of reproducibility, 'learning' effects being practically eliminated by this method.

By repeated tests during a period of several months both subjects were found to possess stable values of final rod adaptation which were identical, namely $1.9-2.1 \log m\mu L$. During part of this period they received a daily supplement of approximately 9000 I.U. of vitamin A, and these values may therefore be considered their minimal rod thresholds.

On certain days the adaptometer was modified to give a test of the 'recovery time' type. The subject was light-adapted to the screen of brightness 2000 millilamberts for a period of 30 sec., and the number of seconds required subsequently in total darkness to perceive the test field of $2^{\circ} 27'$ diameter, and of a constant brightness of $3.5 \log m\mu L$, was measured. As in the majority of such tests no attempt was made to fixate the fovea of the subject. Readings were made in groups of three, with a rest of 3 min. between each reading, over a period of 4 weeks. No 'learning' effect was detected in either subject. It was found that D. S. required consistently a longer period to perceive the test field than D. D., and that the range of variability of both subjects was considerable. The results of fifty successive readings from these two subjects are analysed in Table 1. The difference between the mean 'recovery times' is considerably greater than the sum of the standard errors, and may therefore

be considered to show a real difference in the initial speeds of dark adaptation of these two subjects under these conditions.

TABLE 1. Comparison of fifty successive 'recovery time' readings for two subjects

Subject	Recovery time in seconds			Standard deviation	Standard error
	Mean	Max.	Min.		
D. S.	31.7	53	23	± 6.35	± 0.8806
D. D.	20.8	59	10	± 9.60	± 1.359

It was considered possible that the speed of dilatation of the pupils during the first few seconds of dark adaptation might be a limiting

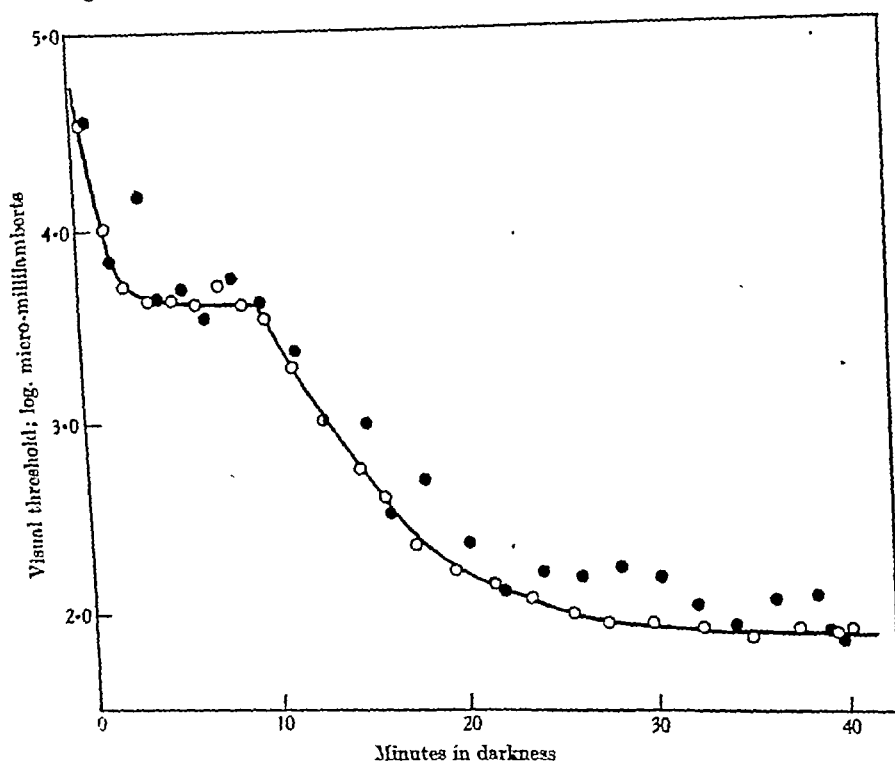


Fig. 1. Subject D. D. The result of one experiment showing the course of dark adaptation of a retinal field $2^{\circ} 27'$ in diameter, following 3 min. light adaptation at 2000 millilamberts, recorded without a fixation light (filled-in circles) and with the test field fixated 6° above the fovea (empty circles).

factor, which would account for the difference between the 'recovery times' of the two subjects, and perhaps also for the large variabilities. The pupil diameters were measured therefore in minimal red light with

a Cruise Scotometer immediately after each reading. Both subjects gave a diameter of 5-6 mm. Independent measurements on D. S. showed that the pupils had opened to this diameter after 15-20 sec. in darkness, at the time when D. D. commonly gave the 'recovery time' reading. A difference in the speed of dilatation of the pupils cannot therefore be the factor determining the difference in the 'recovery times' of these two subjects.

The effect of the lack of a fixation point was investigated in the following way. The course of dark adaptation of both subjects was recorded by the adaptometer method for a period of 40 min. in darkness, following a 3 min. period of light adaptation, first without any fixation point and then with the test field fixated 6° above the fovea. Both subjects gave the same type of results at two sittings made on different days. The results of a single experiment on D. D. are shown in Fig. 1. It is obvious that the recording of dark adaptation is more accurate when the test field of the retina is fixated. The data follow a more steady course, and there is less variability between successive readings. It will be noted also that the readings made without a fixation light indicate in general a poorer level of dark adaptation, which is most apparent in that part of the curve where adaptation of the rods is taking place. This is probably due to the fact that many of the readings made without a fixation point are perceived on the fovea or very close to it, in which part of the retina rods are scarce or totally absent. These curves show clearly that it is important to define the area of the retina tested in order to obtain accurate data at any stage of dark adaptation.

DISCUSSION

It has been shown that for subjects D. S. and D. D. the 'recovery times' required to perceive a field of constant brightness is significantly different, whereas their final rod thresholds are identical and their level of vitamin A nutrition optimal. There is no ground therefore for stating that the longer 'recovery time' of D. S. indicates a poorer state of vitamin A nutrition than D. D. It is emphasized also that the initial speed of dark adaptation bears no necessary relation to the final level of adaptation of the rods, which is the most sensitive index of vitamin A deficiency, although there is probably a general correlation between these two functions [unpublished observations]. Hecht & Mandelbaum [1939] found a partial correlation of 0.44 between the rod and cone threshold values of 110 subjects in good health.

It is concluded therefore that although a test of the 'recovery time' type will probably detect a severe case of night-blindness, such tests are not sufficiently sensitive to give unequivocal data on the course of dark adaptation. This is due partly to the fact that the early stages of dark adaptation are the least affected in states of nutritional deficiency and are the most difficult to measure precisely [Hecht & Mandelbaum, 1940], and partly to the lack of a fixation point to define the position of the retinal field tested. On theoretical grounds moreover it is inadvisable to base a test for vitamin A deficiency on single readings made during that part of dark adaptation which is concerned with the cones, since the relation of vitamin A to the photosensitive pigments of the cones is obscure.

In certain circumstances a 'recovery time' test may be of use. It may be important to measure the initial speed of dark adaptation of aviators, seamen or other nocturnal workers. Such tests may be of value in these cases, especially when the complete course of dark adaptation is recorded also by an accurate method, but they must be considered *ad hoc* tests of the speed of dark adaptation and not a method of diagnosing vitamin A deficiency.

SUMMARY

1. The complete course of dark adaptation and the final level of adaptation of the rods of two well-nourished healthy subjects has been determined, and found to be the same.

2. On the other hand a 'recovery time' type of test, which has been done on each subject fifty times, shows a significant difference between them.

3. The significance of these findings is discussed in relation to vitamin A and the visual cycle, and it is concluded that tests of the 'recovery time' type are of little value for detecting states of vitamin A deficiency.

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THE BEHAVIOUR OF THE PREGNANT UTERUS OF THE GUINEA-PIG

BY G. H. BELL

From the Institute of Physiology, University of Glasgow

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THE factors involved in determining the onset of parturition are certainly complex, but an initial simplification can be made if we assume—and there are good grounds for doing so—that they are chiefly hormonal and that the nervous system plays a relatively minor part. In attacking this problem of uterine activity the first step must obviously be the investigation of the spontaneous activity of the uterus at various stages of pregnancy; and in view of the current theories of the role of the posterior lobe of the pituitary gland the reactivity of the uterus to oxytocin should be examined at the same time. The second step should be the investigation of the oxytocic power of the blood throughout pregnancy; but the difficulties here are so great [see Bell & Robson, 1935] that only the first step is as yet possible.

The purpose of this paper is to describe the spontaneous activity and reactivity of the guinea-pig uterus during and after pregnancy. These have been examined in considerable detail *in vitro* in the human subject [Robson, 1933*b*], in the rabbit [Knaus, 1927, 1928 and Robson, 1933*a*], in the mouse [Robson, 1934], and in the rat [Brooksby, 1937]. Reports of the activity and reactivity of pregnant uteri *in vivo* are much scarcer, much less complete, and are difficult to locate because the abstracts of reviewers often fail to indicate whether the experiments were carried out *in vivo* or *in vitro*. As some early experiments comparing behaviour *in vitro* and *in vivo* in the rabbit [Robson, 1935] showed no marked divergence, perhaps this neglect is not to be wondered at. The human uterus has been studied *in vivo* by Bourne & Burn [1927] and Moir [1934], the rabbit uterus by Knaus [1926] and by Reynolds & Firor [1933] (motility only), and the cat uterus by Robson & Schild [1938]. The information concerning the behaviour of the pregnant human uterus *in vivo* is still fragmentary.

METHODS

It was not found practical to obtain pregnant guinea-pigs by merely putting a male with the female at oestrus. This is not surprising since it is now known [Blandau & Young, 1939] that the period during which the ovum can be fertilized is very short. Each male was allowed to run with three or four females in a single cage; the females were examined once daily and the times at which the vaginae were open were recorded. The vaginae were open for 3 days on the average, with a range of 1-7. The beginning of pregnancy was reckoned as occurring at the middle day of the last oestrous period; the error involved in this assumption is small. If a high proportion of pregnancies is required it seems to be essential to handle the animals as little as possible. Occasionally the vagina opened during a pregnancy, but this did not lead to any confusion, as after some experience it was possible to detect a pregnancy by palpation from 3 weeks onwards and to estimate roughly its duration. Ishii [1920] has described a swelling of the external genitalia with some secretion in ten out of twenty pregnant guinea-pigs which occurred usually about 15 or 30 days after mating. It may be that the opening of the vagina during pregnancy is associated with a wave of follicular growth as described by Loeb [1911]. It seems that the fundamental sex rhythm is not completely suppressed during pregnancy in the guinea-pig; this might also be said of man and *Macacus*, where menstruation (though not strictly analogous with oestrus) is occasionally observed in early pregnancy.

At the time of the experiment each animal was anaesthetized with ether followed by chloralose (7 mg./kg. subcut.) repeated as required. A large number of anaesthetic deaths occurred in early pregnancy, but animals more than 1 month pregnant rarely gave any trouble. The external jugular vein was cannulated; the abdomen was opened and a boat-shaped cannula [Bell & Robson, 1936] was attached to the pregnant horn of the uterus without disturbing the foetus. The movements were recorded on smoked paper by a lever connected by a thread running over two pulleys to the centre of the portion of the uterus under the cannula. In the early pregnancies the cannula was chosen so that it spanned one foetus without compression or alteration of the natural position of the uterus; towards the end of pregnancy a cannula 6 cm. long was applied to the uterus over one foetus—thus a record of only a part of the muscle enclosing the gestation sac was obtained. The lack of standardization is more apparent than real because both in early and in late pregnancy a record of only a sample of the uterine muscle is obtained; if the results of

the investigation of numerous samples are consistent the possibility of error is immensely reduced. A similar method—the Cushny myograph—has already been used by Robson & Schild [1938] to investigate the pregnant uterus of the cat; indeed, some such method is the only feasible one if the pregnancy is to be undisturbed during the experiment. That the vitality of the foetus is little disturbed is proved by one experiment of the present series in which the foetuses showed respiratory movements when they were removed from the uterus at its termination. It is difficult, if not impossible, to estimate the resting length of smooth muscle exposed at laparotomy, but every effort was made to keep the tension of the uterine muscle as constant as possible. This was done, when the abdomen was closed, by observing through the glass cannula the amount of raising of the centre point of the uterine muscle by the thread attached to the writing lever. The body temperature was measured by a thermocouple placed in the abdomen, and no observations were made till that was 38° C. The animals remained in apparently good condition for several hours; this was almost certainly due to the complete closure of the abdomen allowed by this procedure. When a satisfactory sample of spontaneous activity had been recorded intravenous injections of specially purified pitocin (kindly supplied by Dr White of Parke, Davis and Co.) were given about 0.5 c.c. of Locke solution followed by about 0.5 c.c. of Locke solution to wash out the cannula. A graded series of injections was given, and the threshold dose was taken as the minimum amount which would produce a small but sustained contraction; usually this was accompanied by an increase in the frequency of the waves so that the pattern of the spontaneous activity was altered. This part of the experiment was not unduly prolonged so that strips of the uterus were obtained in good condition for the experiments *in vitro*. Two strips were taken from the portion of the uterus to which the cannula had been attached and were suspended in the usual thermostatically controlled bath containing 60 c.c. of oxygenated Locke solution.

RESULTS

Typical records from the living animal are shown in Figs. 1 and 2, from early and late pregnancy respectively. The uterus was active, as in other animals, at all stages of pregnancy, and in general the amplitude of the movements increased with the duration of pregnancy, i.e. as the amount of muscular tissue increased. The pattern of the contraction waves in any one experiment is not by any means regular and it is not easy to distinguish tracings (except by amplitude) made early in preg-

nancy from those made towards the end; the mean duration of the contraction waves up to the 30th day was 3.6 min., from the 31st to the 49th day it was 4.7 min., and from the 50th to the end of pregnancy it was 10.4. Owing to the scatter of the observations only the difference between the very early wave durations and the parturient values are significant (2.23 times the standard error).

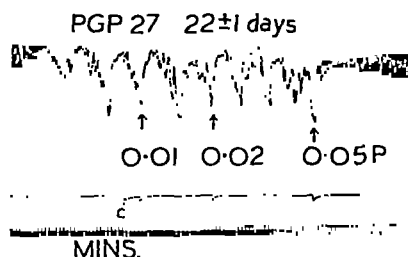


Fig. 1. *PGP 27*, 22 ± 1 days pregnant. Reaction to 0.05 unit of pitocin intravenously; no reaction to smaller doses. At *C* chloralose given subcutaneously.

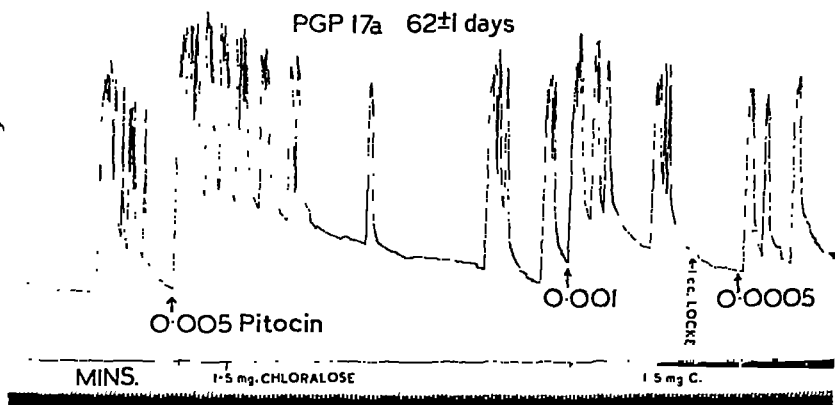


Fig. 2. *PGP 17a*, 62 ± 1 days pregnant. Reaction to 0.001 unit of pitocin intravenously; smaller doses of pitocin and control dose of plain Locke solution without effect.

The threshold dose of oxytocin at various stages of pregnancy is recorded in Fig. 3. Because of the wide range of values the dosage has been plotted on a logarithmic scale; this spreads out the lower values and makes their significance clearer. It will be seen that in early pregnancy the uterus is comparatively unreactive, but that it becomes more and

more reactive as the pregnancy proceeds. During the last fortnight the uterus reacts to one-fiftieth of the amount required in early pregnancy.

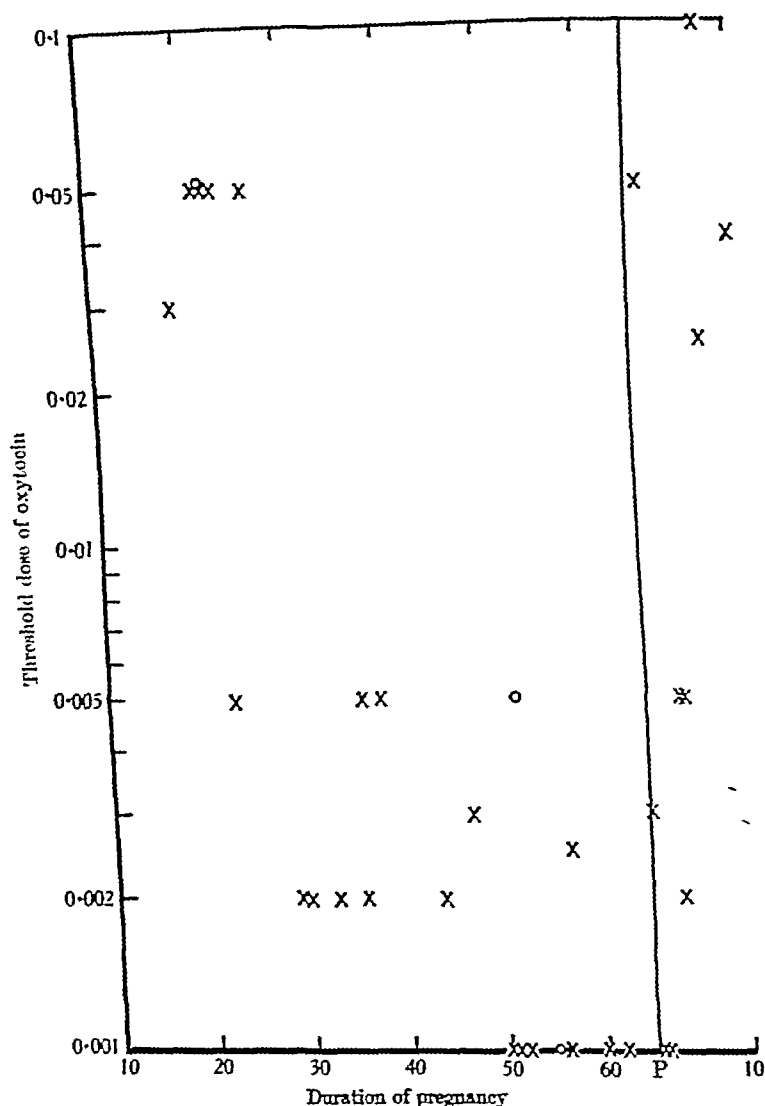


Fig. 3. The threshold dose of oxytocin at various stages of pregnancy. x denotes that the duration of pregnancy was timed from the opening of the vagina; o means that the duration of pregnancy was estimated from the graph of Fig. 4.

Very soon after parturition the reactivity becomes once more much less than the prepartum level. There is some variation in the actual weight

of the animals (see Discussion) at the time of experiment even when allowance is made for the weight of their foetuses. The greatest correcting

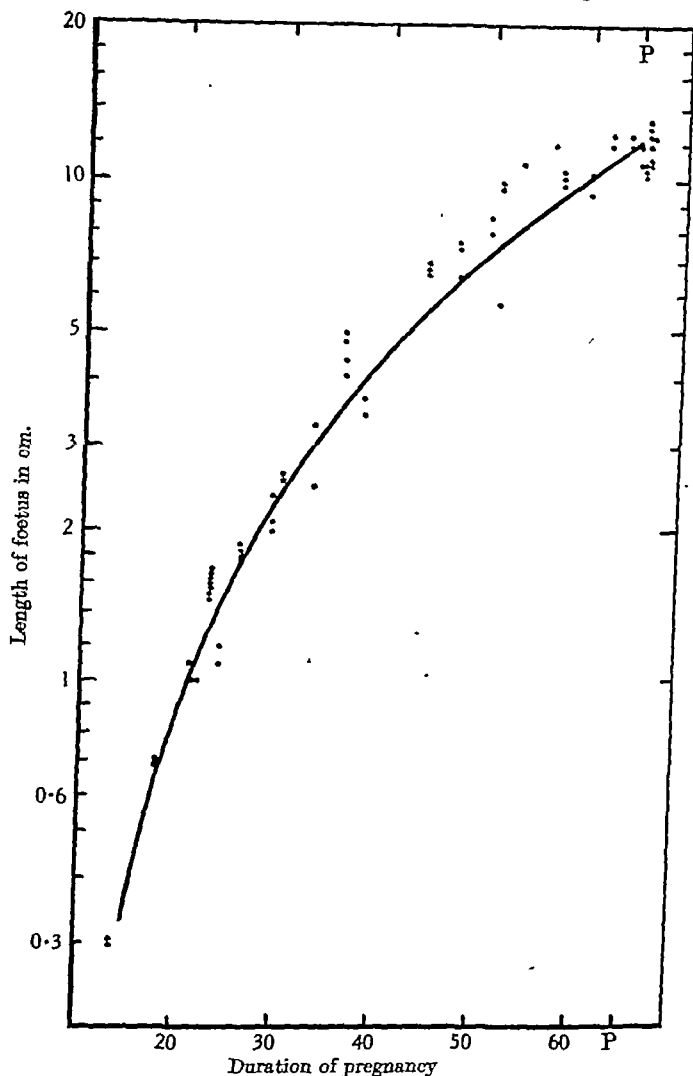


Fig. 4. This graph shows the rump to nose lengths—plotted on a logarithmic abscissa—of all foetuses whose age was known. The line has been drawn to the equation

$$y = -0.417 + 0.00407x^{1.224},$$

where y is the length of the foetus in cm. and x the duration of the pregnancy in days.

factors necessary to express the threshold dose on the basis of the average body weight are 1.5 and 0.7; in most cases it would be, of course, much

less. This is within the range of the experimental error in determining the threshold and therefore the correction has not been made. In any case it would alter very little the general trend of Fig. 3.

When the threshold doses from the *in vitro* experiments are plotted on a diagram like Fig. 3 there is a very wide scatter of points, and as will be shown in the Discussion very little reliance can be put on them.

The weights of the foetuses in any one litter were very variable, whereas the lengths were remarkably similar. An attempt to find a formula to give the average weight of a litter corrected for the number in it had to be abandoned for lack of information. Fig. 4 gives on a logarithmic ordinate the observed length (nose to rump) of all the foetuses against the duration of pregnancy on the abscissa. The continuous line has been drawn to the equation $y = -0.417 + 0.00407x^{1.424}$, where y is the length of the foetus in cm. and x the duration of the pregnancy in days. This is the simplest form of equation to give a reasonable fit; no physiological significance is to be attached to the constants, the equation obviously cannot hold in early pregnancy and must not be used at durations less than 15 days. This graph (Fig. 4) was used to estimate the duration of pregnancy in some animals; the data obtained in this way are entered as \circ in Fig. 3; an \times in Fig. 3 indicates that the duration of pregnancy is reckoned from the vaginal opening. The small degree of scatter in Fig. 4 suggests that timing by vaginal opening is reasonably accurate.

DISCUSSION

Pregnancy in the guinea-pig is maintained as in other species in spite of active movements of the uterus. The results support the theory that parturition is dependent on sensitization of the uterus to oxytocin, but since the uterus shows a high reactivity to oxytocin during the last fortnight of pregnancy this sensitization by itself cannot be enough to bring about parturition. The actual moment of delivery might be determined by a sudden outpouring of oxytocic material. The guinea-pig can maintain a pregnancy and deliver its young after removal of the pituitary gland [Pencharz & Lyons, 1933]; the source of the oxytocic material in a normal delivery is thus not necessarily the pituitary—it may be the hypothalamus, the placenta, or even the foetal pituitary [Bell & Robson, 1937]. This notion of the cause of parturition could be confirmed only if information of the oxytocic property of the blood at various stages were available. But this is an elusive problem which will not be solved until we have a specific test for oxytocin. The difficulty is that the uterus of any animal contracts in the presence of a large number of substances

many of which have obviously no physiological significance [Bell & Morris, 1934; Bell & Robson, 1935].

Before proceeding to discuss the cause of the alteration in reactivity to oxytocin certain fundamental questions must be considered, namely, the manner in which the pitocin reaches the uterus and its concentration when it does so. Since the injection into the jugular vein was always given within a few seconds and in approximately constant volume into an animal with a very short circulation time the oxytocin should be distributed rapidly and evenly through the blood stream; the rate of arrival or impact on the uterus should be about the same in different experiments, always provided that the circulation rate through the muscle remains the same throughout pregnancy. It is well known, however, that the amount of blood in the uterus and the rate of blood flow through it increase as pregnancy advances; but since at the same time the mass of the uterus becomes greater, there may be little alteration in the circulation rate per unit volume of muscle. More important still is the finding [Barcroft & Rothschild, 1932] that the blood volume in the uterus is most closely related to the weight of the placentae; the increased blood flow is needed to meet the requirements of tissues other than the myometrium. Although there is no quantitative information there seems to be no *a priori* reason to expect that the myometrium during pregnancy—when there is no great activity—should require an increased blood flow; furthermore, it would require a 50-fold increase of vascularity from the 20th to the 50th day of pregnancy to reduce the data of Fig. 3 to a common level. It seems only reasonable then to assume that the alteration in reactivity observed does indicate a real alteration in the state of the uterus. This perhaps academic discussion does not at all affect the main conclusion that during the last fortnight of pregnancy a relatively small quantity of oxytocin is able to produce a marked effect on uterine activity and that parturition could be much more easily initiated than early in pregnancy.

There remains the explanation of the variation of the sensitivity of the uterus to oxytocin throughout pregnancy. According to Loeb [1906, 1911] the corpus luteum of the guinea-pig is fully formed about 5 days after ovulation and remains till the 40th day when degenerative changes are found in it. It will be seen from Fig. 3 that this marks the middle of the transition period from very low to very high reactivity of the uterus to oxytocin. It has been known for many years that the guinea-pig goes into heat shortly after parturition, and more recently that ovulation with subsequent formation of corpora lutea takes place at this time [see

Parkes, 1929]. The rise in the reactivity of the uterus towards the end of pregnancy is associated in the present experiments with the decline of the corpora lutea of pregnancy, and the post-partum fall in the reactivity to oxytocin is associated with the formation of fresh corpora. The results of Bell & Robson [1936] were taken to show that 'progesterin has no appreciable inhibitory action either on the reactivity to oxytocin or on the spontaneous rhythmic activity of the guinea-pig uterus'. At the time of these experiments pure progesterone had just become available in small quantities, and the technique did not allow of so accurate an estimate of the threshold dose as that used in the present series of experiments. The effect of larger doses of progesterone is being investigated at present because if larger doses of this hormone cannot bring about a reduction in reactivity it will be necessary to postulate the action of some other hormone. It may be that the high reactivity at the end of pregnancy is brought about by the decline of luteal activity and at the same time increased oestrin action. The results of Bell & Robson [1936], however, did not show any increase of reactivity in oestrin-treated animals.

The degeneration of the corpus luteum about the 40th day of pregnancy is strikingly confirmed by Pencharz & Lyons [1933], who found that hypophysectomy on the 35th day terminated the pregnancy, while if it were performed on the 41st day it did not cause abortion. Under these circumstances one would expect an immediate degeneration of the corpus luteum, and in fact they found that immediately after delivery the corpus luteum was markedly degenerated. There is very good evidence, however, that the placenta of some animals can produce sufficient progesterone to maintain a pregnancy after ovariectomy [for discussion see Robson, 1940]. This does not seem to have been shown in the guinea-pig—perhaps because evidence of progesterone action cannot readily be obtained in this animal. The present series of experiments fits in well with previous work suggesting the decline of the luteal activity about the 40th day and gives no evidence to show that progesterone is supplied by the placenta.

If this theory of a sudden outpouring of oxytocin is a true explanation of the occurrence of parturition it is difficult to see why a diminution of the reactivity to it in early pregnancy is necessary. It might be argued that, since the outpouring of oxytocin is the actual determining factor the early low reactivity may be an accidental finding. Marrian & Newton [1935] have suggested that, while the uterus is growing, its metabolism may be so altered that it responds more readily to oxytocin. If the data of Fig. 3 are plotted on evenly divided co-ordinates it is found that the

foetus, and therefore presumably the uterus, is growing most quickly when the sensitivity is rising. Curiously enough exactly the opposite occurs when the uterus is involuting; after parturition the reactivity declines very quickly, in some cases it is very low even before the corpus luteum is formed. In the pregnant guinea-pig low reactivity is associated with small fibres—presuming that an increase of uterine size is brought about mainly by an increase in the size of the fibres. Bell & Robson, however, showed that administration of oestrone with or without progesterone produced a small increase in the size of the uterus without alteration of reactivity in the non-pregnant animal; obviously this also requires further investigation. A point in support of this theory of sudden outpouring of oxytocin is that it would explain satisfactorily the finding of Loeb [1923] and Herrick [1928] that removal of corpora lutea in the guinea-pig did not always result in the termination of pregnancy.

In the present work, the marked difference between the concentrations of oxytocin necessary to produce contraction of the uterus *in vivo* and *in vitro*, already described for the non-pregnant guinea-pig [Bell & Robson, 1936], has been confirmed. The weights of the pregnant animals varied from 430 to 904 g., with an average of 599 g.; the average blood volume may be taken as 40 c.c. Using this information, it was found that the ratio of the threshold concentration in the blood (i.e. *in vivo*) to the threshold concentration in the bath of 60 c.c. (i.e. *in vitro*) varied from 0.1 to 50 with an average of 3.6. Although the discrepancy is less in the present series than in the previous one the conclusion is still that the reactivity of the guinea-pig uterus *in vitro* is a very unreliable guide to its reactivity *in vivo*; indeed, it is quite likely that there is no relationship between the *in vivo* and *in vitro* findings—in the twenty-five cases in which both experiments were performed the correlation coefficient is +0.24 with a standard error of 0.20. Furthermore, the type of spontaneous movements given by uterine strips *in vitro* is quite different to that seen *in vivo*; the ratio of the duration of spontaneous waves *in vivo* to the duration of spontaneous waves *in vitro* varied in the present series from 0.2 up to 10. In spite of doubts which have been raised [Bell, 1941] concerning the reliability of observations made by levers producing tension on the uterus, and in spite of the well-recognized difficulty of the effects of anaesthesia, one is still inclined to believe that records made from the living animal are more likely to represent the actual condition of affairs in the undisturbed animal than are records obtained *in vitro*. The discrepancies found between the *in vitro* and *in vivo* results in the cat forced Robson & Schild [1938] to the same conclusion, which they expressed thus: 'in

certain species it is necessary to investigate the spontaneous activity and the responses of the uterus in the intact animal in order to assess in a satisfactory manner the effects of hormones on the uterus.'

SUMMARY

The activity of the guinea-pig uterus in vivo was examined at various times during and after pregnancy. Spontaneous activity occurred at all times. The threshold dose of oxytocin required to elicit a contraction is high at the beginning of pregnancy and becomes much less in the last fortnight; it rises again shortly after parturition. The threshold dose declines about the time at which the corpus luteum is known to degenerate and increases again when a new corpus luteum is formed. The information at present available does not allow of any explanation of this behaviour in terms of oestrin or progesterin. The actual moment of parturition cannot be determined by high uterine reactivity to oxytocin alone, but possibly by a sudden outpouring of oxytocin at a time when the uterus is highly sensitive to it.

A graph with an approximate equation for the estimation of the age of guinea-pig foetuses from their length is given.

The behaviour of the guinea-pig uterus in vitro is an entirely unreliable guide to the behaviour of that organ in the intact animal.

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A QUANTITATIVE METHOD OF ASSAY FOR THROMBIN AND PROTHROMBIN

By L. B. JAKUES

*Departments of Physiology and Physiological Hygiene,
University of Toronto, Toronto, Canada*

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DIFFICULTIES in developing quantitative methods have greatly hindered studies in blood clotting. Two methods [Jaques & Charles, 1941] are available for the biological assay of coagulant and anticoagulant substances, namely (1) the determination of the amount of active substance which gives a fixed clotting time, and (2) the determination of the clotting time with a given amount of the substance. A thrombin unit has been defined by Mellanby [1933] and by Warner, Brinkhous & Smith [1936]¹ by the first method. While it is advisable to define the unit of activity in absolute terms, the many well-known advantages of using a standard preparation to define a unit of activity have led to the widespread adoption of this practice in most biological assays, and to the use of the comparative assay (the comparison of the potencies of the standard and unknown under identical conditions). Using a thrombin preparation (standardized in thrombin units under the conditions established by Mellanby or Seegers) as reference standard, it is possible to use either of the above methods of assay as the basis of the comparison. The preparation used in the present work was standardized in terms of the Iowa unit.

For the assay of thrombin, the second method of assay (the determination of clotting time) has several practical advantages. Thus it is applicable to a wide range of thrombin concentrations (does not require dilution to a specific activity). Further, the determination of clotting times can be made the basis of a 'quantitative' assay, i.e. an assay in

¹ Seegers [1940] has pointed out that, while the unit as used and defined at Iowa is based on a different clotting time and clotting system to that used by Mellanby, the activities of the units defined by the different methods have been found to be essentially the same.

which an arithmetical value is obtained from each tube. However, in order to convert these clotting times to thrombin concentrations, it is necessary to establish the relationship between the two. Various authors [e.g. Barratt, 1937; Quick, 1936] have suggested different forms. It has been found empirically by the author that a straight line is obtained by plotting the logarithms of the clotting time and thrombin concentration. It should be noted that Barratt's and Quick's equations can be reduced to this form. A linear relationship greatly facilitates the comparison of the potency of the unknown and standard, and it also reduces the experimental error to the same order throughout the range of determined values.

Either fibrinogen solutions or decalcified plasma can be used for assaying thrombin, since each give a linear relation between concentration and clotting time. While, owing to the antithrombin present, plasma cannot be used to detect traces of thrombin, it appears to be somewhat more sensitive to changes in thrombin concentration over the higher range. Plasma is fairly constant in composition, but to prepare purified fibrinogen and to adjust the solutions so as to have an identical composition each time is by no means a simple procedure. A further advantage of plasma is that the fibrinogen contained in it is relatively stable. Thus citrated horse plasma kept for 4 years without any special precautions was found to yield a good firm clot with thrombin. Solutions of fibrinogen, on the other hand, require the addition of colloid before they give a firm clot and, after a few days' storage in the ice-box, precipitate rather than clot on the addition of thrombin. For these reasons, plasma appears to be preferable as a test material.

For prothrombin assays, the presence of this substance in the plasma offers a difficulty. It may be removed by alumina gel [Quick, 1936]. Providing suitable controls are conducted, plasma containing prothrombin may be used. Citrated horse plasma, particularly when aged for a month or so, is clotted only slowly by thromboplastin prepared from ox lung or rabbit brain; it is, therefore, quite suitable for prothrombin assays without any further treatment.

METHODS AND RESULTS

Prothrombin was prepared by Mellanby's method [1933] and then converted to thrombin and purified as described by Seegers. It was kept in the form of a dry powder in the ice-box. Dr Seegers kindly assayed a sample of the lot used as reference standard, and reported that it contained 49 (Iowa) units per mg. It was then reassayed by the method

described here, using as standard a sample of thrombin supplied by Dr Seegers and containing 200 units per mg. The potency of the sample by this method was 50.6 units per mg., i.e. the average of the two results was 50 units per mg. As the sample was completely soluble in a concentration of 1 mg. per c.c. and did not lose activity in solution on keeping for a week in the cold, it fulfilled the requirements of a satisfactory standard. For use, it was dissolved in isotonic saline, containing 0.3% tricresol and 1% (by volume) imidazole buffer (Seegers). A fresh sample was weighed out every 3 or 4 days as required. Citrated horse plasma (100 c.c. 8% trisodium citrate to 1 l. blood) was obtained from normal horses. As an antiseptic, 0.9% ether-phenol (1 : 1) mixture was added. This was not essential, as the oldest plasma tested (4 years) contained no preservative. When horse plasma is not available, aged citrated plasma from other species can be used.

To 0.3 c.c. plasma in test tubes 8 mm. diameter (selected for uniform bore) is added 0.10 c.c. imidazole buffer (diluted 1 : 10 with saline), and finally the thrombin solution (usually 0.2 c.c.). The total volume is previously adjusted to 0.6 c.c. with saline if necessary. (The use of the buffer is not essential, but does appear slightly to improve the accuracy of the assay.) The tubes are immersed in a bath at 20° C. and examined by tilting at frequent intervals. To measure the clotting time, a stopwatch (placed face downwards on the desk) is used. The point of transition from the fluid to the gel state is taken as the end-point. This is easily observed when the tube is tilted slightly, as the surface of the plasma becomes rigid at this point.

When the prothrombin content of plasma is to be determined, the fibrinogen and antithrombin present in the sample decrease the yield of thrombin from the activation process. Warner *et al.* have developed methods to overcome this difficulty. The sample is cleared of fibrinogen by their ingenious method, using thrombin. Two units of thrombin are added to 1 c.c. plasma and the latter defibrinated with a glass rod. It is allowed to stand 15 min., any fresh fibrin removed and a second 2 units added. This usually removes all residual fibrinogen. The effect of antithrombin is largely overcome by diluting the plasma a hundredfold in the activation mixture. For the assay, several activation mixtures, with different dilutions of the prothrombin of this order, are set up. The activation mixtures contain 0.3 c.c. diluted plasma (1 : 20-1 : 50), 0.1 c.c. dilute imidazole buffer (1 : 10), 0.3 c.c. thromboplastin [cf. Seegers], 0.1 c.c. 1% calcium chloride and saline to 1.2 c.c. All dilutions are made in 0.85% saline containing 0.3% tricresol as preservative. For pro-

thrombin solutions, suitable dilutions are chosen, so that the mixture contains between 0.03 and 3.0 units in 0.2 c.c.

Standardization of the plasma. Each sample of plasma used as a source of fibrinogen is standardized with standard thrombin, using varying amounts of thrombin (0.2–5.0 units) such as to give a series of clotting times from 10 sec. to 10 min. The data are then plotted on logarithmic

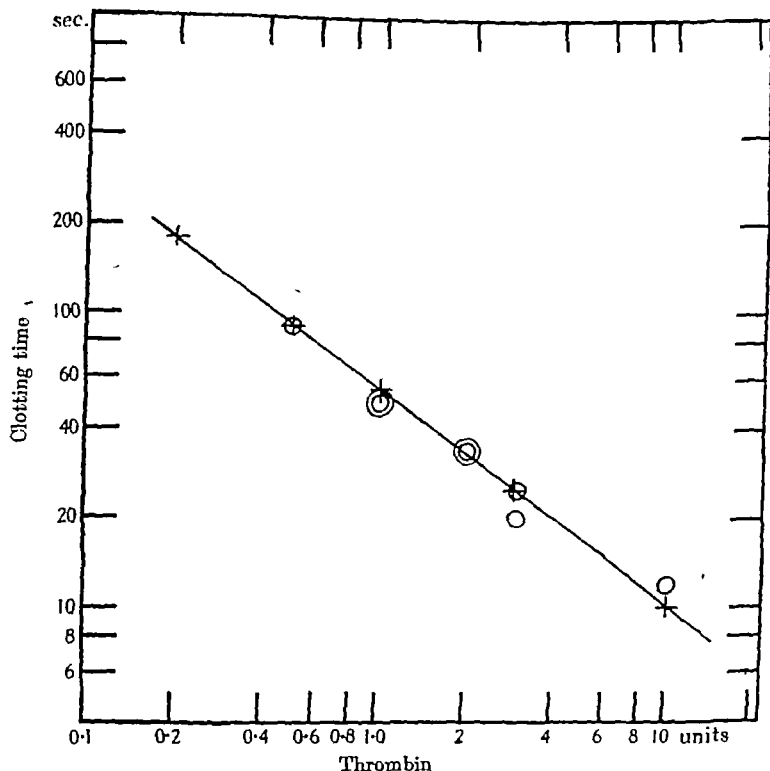


Fig. 1. Standardization curve of plasma collected 7 November 1940. O Determined 3 December 1940; + determined 10 December 1940.

paper (clotting time in sec. against thrombin concentration in units) and a straight line is drawn to give the best fit. Such a standardization graph is shown in Fig. 1. As can be observed, a linear relationship is obtained between thrombin concentration and clotting time for clotting times from 10 sec. to 10 min. The linear relationship appears to hold beyond these limits also. The plasma when tested with calcium and excess thromboplastin gave a clotting time of 5 min. (fresh plasma, 2½ min.) and its prothrombin content by titration was 210 units per c.c. The

standardization was repeated 1 week later. The plasma had been kept in the ice-box during this period but had been removed to the room for assays on several days. It can be seen that the standardization on the second occasion gave a set of results which were strikingly similar to those of the first. The close agreement between the two justifies the use of the standardization graph for determining the potency of unknown thrombin solutions.

The assay of thrombin. To determine the potency of an unknown thrombin solution, the clotting time is determined with several concentrations of the unknown and its potency then calculated from the graph. The latter is checked each day with several concentrations of the standard thrombin, and as soon as such samples give clotting times which deviate from the original curve, the plasma is either restandardized, or discarded and a new one standardized. The use of a standardization graph, while not achieving the greatest accuracy possible with the method, would appear to be sufficiently accurate for most clinical and research purposes, e.g. to follow the activation of prothrombin in assays for the latter, the inactivation of thrombin by antithrombin, and similar problems.

When maximum accuracy is required, as when establishing a second standard in terms of the first, or accurately comparing the potency of two thrombin preparations, it is advisable to use an assay designed on statistical principles, such as has been described by Bliss & Marks [1939] for the biological assay of insulin and by Bliss [1940] for the assay of vitamin D. A modification of the method has therefore been developed for this purpose. In a biological assay, using a graded response such as this, the potency of several concentrations of the unknown should be compared with an equal number of concentrations of the standard. In order to avoid bias due to treatment, etc., it is necessary to randomize the samples [cf. Fisher, 1938]. Then, knowing the relationship between response and dosage (concentration), it is possible both to determine the potency of the unknown in terms of the standard and to calculate the error. Bliss & Marks, and Bliss, have described the application of factorial design and analysis to such assays. This provides a simple method for the treatment of the data and further provides a means of testing in the actual assay for any divergence from the linear relationship or parallelism of the two dosage-response curves. Their methods have been satisfactorily applied to the assay of thrombin. As an example of the application of the method, the results of an experiment will be described, in which a solution of the standard was taken as the unknown

and compared against itself as standard. This allows one to compare the known potency with that determined experimentally. 0.5, 1.0, 2.0 units of standard and 0.01, 0.02, 0.04 mg. of the unknown were chosen, and dilutions containing these in a volume of 0.2 c.c. were made. The clotting times were then determined with these amounts of standard and unknown, three complete sets of the various dilutions being carried out. In order to avoid any bias due to order of mixing, etc., the dilutions were taken in the order of a Graeco-Roman square [see Fisher]. The experimental values are shown in Table 1. These were converted to their

TABLE 1. Clotting times in sec.

Set no.	S_1	S_2	S_3	U_1	U_2	U_3
1	41	60	97	36	62	99
2	36	63	96	35	70	108
3	38	58	112	39	62	114

Log of dosage interval = 0.3010.

logarithms and the several treatment factors isolated by the factorial scheme of Bliss & Marks. The log-ratio of potencies, M , was found to be 0.0139 ± 0.0179 . Converting to original units and assuming the standard to be 50 units per mg., the potency of the unknown was 51.63 ± 2.12 units per mg. (theoretical potency, 50.0).

The assay of prothrombin. A number of methods for the assay of prothrombin are in use [Brinkhous, 1940]. The one-stage methods require an arbitrary standardization curve [Quick, 1939]. In the two-stage method of Warner *et al.* the prothrombin is activated to thrombin and the potency of the latter assayed. The latter authors have pointed out that this method has the advantage that it distinguishes a slow rate of activation of the prothrombin from a lowered prothrombin content, whereas these are not distinguished in the one-stage method. A useful modification of the two-stage method would appear to be the assay of the thrombin by the method described above. Warner *et al.* assay the thrombin by the dilution method with a fixed clotting time. Hence it is necessary in their method, as originally described, to activate a number of dilutions of prothrombin to find the dilution which yields one unit of thrombin. Further, to follow the activation process with time, involves such a large number of clotting time determinations that it is not possible as a routine. Since with the method described here each clotting time obtained can be converted to units of thrombin, an actual activation curve for each prothrombin dilution can be easily determined. This allows one to distinguish such effects as an increase in the rate of inactivation of the formed thrombin as a routine practice. The method

also reduces the actual number of activation tests required. By simply determining the activation curves for two dilutions of prothrombin, it is possible to do the assay in duplicate and to ensure that the maximum number of thrombin units are formed. Brinkhous has pointed out that one of the difficulties with the two-stage technique is the preparation of the reagents, especially the clotting mixture. This is easily overcome by the use of plasma.

TABLE 2. Assay of plasma prothrombin

Final dilution of plasma	1 : 450			1 : 150	
	Activation time min.	Clotting time sec.	Thrombin units	Clotting time sec.	Thrombin units
	5	244	0.09	128	0.20
	10	217	0.11	94	0.29
	15	210, 211	0.11	92	0.30
	20	199	0.11	73	0.39
	25	173	0.14	81	0.35
	30	191	0.12	86	0.32
	35	182	0.13	88	0.31
	40	—	—	90	0.30
	45	—	—	82	0.34
	Average = 0.12			0.33	

Prothrombin content of plasma was 270 and 250 (average 260) units per c.c. Subject, B. H. Sample of blood decalcified with 1/10 volume N/10 sodium oxalate.

The method has been chiefly used to follow the activation of prothrombin solutions and has been found quite satisfactory for these. It has also been used to determine the prothrombin content of plasma. This is illustrated in Table 2 for human plasma. The activation was conducted at two dilutions of the defibrinated plasma (see Methods, p. 276 above) and the thrombin assayed by adding 0.2 c.c. activation mixture to 0.3 c.c. plasma + 0.1 c.c. dilute buffer, the potency being calculated from the standardization graph.

SUMMARY

A rapid quantitative method for the assay of thrombin is described. It is based on the finding that a linear relationship is obtained when the clotting time is plotted against thrombin concentration on logarithmic paper. Citrated horse plasma and a standard thrombin preparation (standardized in thrombin units according to Seegers) are used. With these a standardization graph is obtained from which the potency of an unknown thrombin solution can be readily determined. The application of the method to prothrombin assays is indicated.

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THE LATENCY AND CONDUCTION OF POTENTIALS IN THE SPINAL CORD OF THE FROG

By FWU TARNG DUN¹

From the Physiological Laboratory, Cambridge

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THE electrical activity in the spinal cord has been observed mainly in two different ways. Gasser & Graham [1933] have used leads placed longitudinally on the dorsal surface of the spinal cord in the cat cephalad to the root stimulated, and recorded the electrical changes in response to stimulation of a dorsal root. The changes consist of a negative spike followed by a longer and slower negative complex which is usually succeeded by a prolonged positive wave. Of these the spike has been attributed to afferent fibres and the succeeding components to internuncial neurones. It is because of this that the latter group of potential changes are denoted by them as intermediary potentials. The same method of observation was adopted later by Hughes & Gasser [1934*a, b*] and Hughes, McCouch & Stewart [1937]. A second method of studying the electrical activities in the spinal cord is to observe the electrotonus they produce in the roots. This method was used by Umrath [1934]. Umrath & Umrath [1934]. Barron & Matthews [1936*a, b*; 1938*a*] and Eccles & Pritchard [1937]. The electrical changes recorded with two electrodes on the root consist of a prolonged negativity of the electrode near the cord. The characteristics of this slow negativity correspond in most respects to those of the positive wave in the records obtained by Gasser & Graham from the dorsum of the spinal cord. Barron & Matthews, however, express the opinion that this slow negativity arises at the terminations of the dorsal root fibres themselves, and not in the internuncial neurones.

The origin of these slow potential changes inside the cord is a problem of importance, for such changes are the only direct signs of physiological processes taking place inside the cord. Unless we know definitely where

¹ Research Fellow of the China Foundation.

these potential changes originate and which of the many physiological processes inside the cord they represent, we cannot hope to make use of them successfully as a means of studying the spinal cord activities.

The present experiments were undertaken to determine the origin of the potential changes recorded in the dorsal roots by observation of the latency of the dorsal root potentials, and the manner of their conduction in the spinal cord.

METHOD

In all experiments *Rana esculenta* were used. The spinal cord was exposed from the dorsal aspect under ether anaesthesia, decerebration was effected by pithing the brain or by section of the brain stem. Care was taken to maintain the circulation of the cord in good condition. The roots prepared for recording the potentials were severed immediately central to the spinal ganglion. The sciatic nerve with the 10th dorsal root and the brachial nerve with the 3rd dorsal root were sometimes dissected free for stimulation and recording. Subcutaneous injections of curare just sufficient to stop limb movements were given. Except at the moment when the records were being taken, oxygenated Ringer was kept continuously dropping on the surface of the cord throughout the experiment.

For the investigation of conduction of the dorsal root potentials in the spinal cord, various spinal cord lesions were produced. The operations were usually made with a razor blade or a pair of sharp scissors at the region of the posterior enlargement between the 9th and the 10th dorsal roots. The preparation was then given at least 3 hours to recover before the commencement of the test. A stretch of cord of about 7 or 8 mm. long containing the lesion was removed after the observations, and a drop of Indian ink was dropped on the surface of the lesion. Then it was prepared with osmic acid and cut into serial sections. The extent of the lesion was examined under the microscope.

The potentials were ordinarily recorded with a balanced input three-stage condenser coupled amplifier and a Matthews' oscillograph. The records obtained were, however, frequently checked with a direct coupled amplifier. For electrical stimulation a coreless coil was used. Silver silver-chloride Ringer electrodes were used with worsted leads to the nerve for recording. Silver silver-chloride electrodes were put directly in contact with the nerve for stimulation.

The speed of the recording camera was 184 mm. per sec. With a microscope it was possible to measure records accurately to 0.05 mm. Time intervals were thus measured to about 0.3 msec.

PART I

The latency of the dorsal root potentials

If a volley of sensory impulses is sent into the cord through the 3rd or 10th dorsal root, the electrical changes inside the cord can be detected in practically all other roots on the ipsilateral side as well as in the stimulated root itself. On the contralateral side potential changes have been recorded in all the thicker lumbar dorsal roots. The amplitude is much smaller than that in the corresponding ipsilateral roots. There are also potential changes in the thinner thoracic dorsal roots on the contralateral side, but they have not been investigated. A sharp beginning of the potential changes is necessary for an accurate measurement of latency, and observation was confined to the potential changes in the ipsilateral roots.

The amplitude of the dorsal root potential is apparently determined by at least three different factors: (1) the size of the root in which it is recorded, (2) that of the root stimulated, and (3) the distance between these two roots. Generally speaking the amplitude of the dorsal root potential increases with the size of the roots but decreases with the distance between them. There may be another factor in the structural relationship between two roots, but this has not been investigated thoroughly.

The latency of dorsal root potentials in roots cephalad to the root stimulated

Fig. 1 shows the potential changes recorded from three different dorsal roots on the right side of the spinal cord when the ipsilateral 10th dorsal root was stimulated. A shows the potential changes in the 9th dorsal root, which is nearest to the root stimulated. B shows the potential changes in the 6th dorsal root. C shows the potential changes in the 4th dorsal root, which is the most distant root in the three. One sees that the 4th dorsal root potential has the longest latency and the 9th dorsal root potential the shortest. The magnitude of the 4th dorsal root potential was smaller than that of the 6th, this was again smaller than that of the 9th. In order to compare the latency the smaller dorsal root potentials were, however, subjected to larger amplifications. So that their recorded size is approximately the same.

Besides the increase of latency it is also of interest to see that the shape of the dorsal root potentials changes gradually as the root in which it is recorded is situated farther and farther away from the root

stimulated. The 4th dorsal root potential reaches its maximum more slowly than the 9th one.

Table 1 shows the latency of dorsal root potentials in roots on one side of the spinal cord when the ipsilateral 10th dorsal root was stimulated.

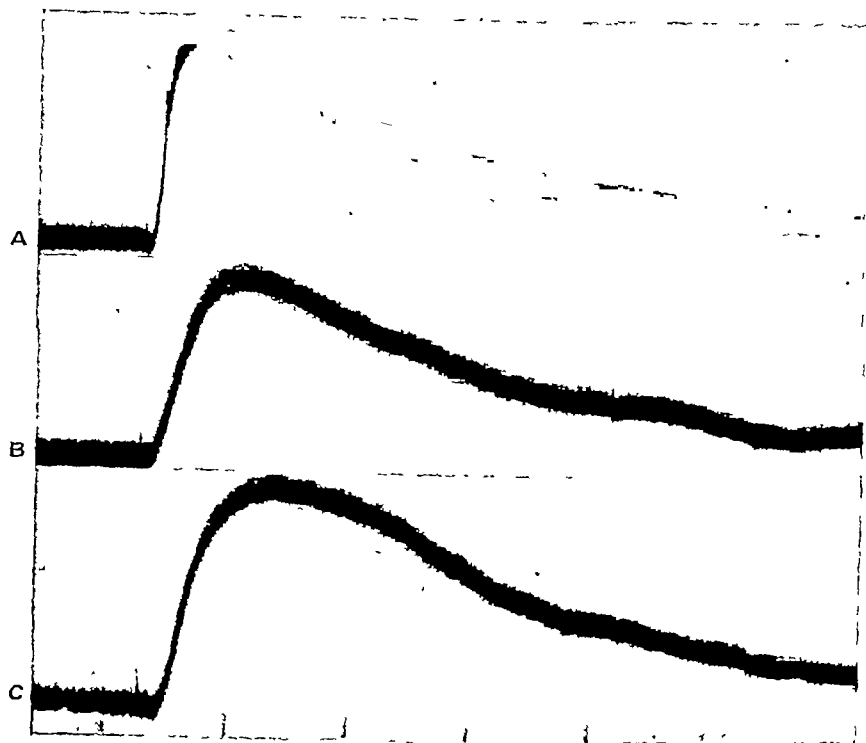


Fig. 1. Showing the increase of latency with the distance between the root stimulated and the root in which it is recorded. A sensory volley of impulses was sent into the cord through the 10th dorsal root. A, the potential changes in the 9th dorsal root, which is 2.2 mm. from the 10th root. B, the potential changes in the 6th dorsal root, which is 8.7 mm. from the 10th dorsal root. C, the potential changes in the 4th dorsal root, which is 13.5 mm. from the 10th dorsal root. Recording electrodes 1 and 7 mm., stimulating electrodes 4 and 4.5 cm., from the cord. 15 0° C. (Time, 0.1 and 0.02 sec.) Direct coupled amplifier.

It contains the results obtained from eight different preparations. For each preparation the distance between the 10th root and the root in which the potential was recorded is given under column D. Under column L are the latencies of different dorsal root potentials. The value of each latency is an average of at least five readings, although they are

TABLE 1*

Root no.	Frog no. 60 L. 16.8° C.		Frog no. 60 R. 16.8° C.		Frog no. 61 L. 15.7° C.		Frog no. 61 R. 15.7° C.		Frog no. 62 L. 15.8° C.		Frog no. 62 R. 16.0° C.		Frog no. 63 L. 16.8° C.		Frog no. 63 R. 16.8° C.	
	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.
4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	9.2	12.3	9.5	13.8	9.5	12.7	9.7	14.0	8.4	15.7	8.4	11.1	13.1	13.8	13.5	—
6	8.1	11.0	8.2	—	8.2	12.5	8.7	12.9	7.2	13.0	7.2	11.0	11.1	13.4	11.2	13.6
7	6.6	9.7	6.4	11.2	6.5	12.4	7.0	10.1	5.6	9.4	5.6	9.7	8.1	9.8	9.5	11.6
8	4.0	8.6	—	—	3.7	10.2	3.8	8.6	3.4	8.6	3.4	7.4	4.0	8.6	4.2	8.0
9	1.8	7.3	2.1	7.6	1.9	8.9	1.3	7.2	1.8	6.0	1.4	6.3	2.5	7.0	2.0	6.4

TABLE 2*

Root no.	Frog no. 71 15.8° C.		Frog no. 72 13.6° C.		Frog no. 73 15.5° C.		Frog no. 75 15.0° C.		Frog no. 92 20.0° C.		Frog no. 93 18.0° C.		Frog no. 94 16.0° C.		Frog no. 95 16.5° C.	
	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.	D mm.	L msec.
4	1.5	7.8	1.2	6.5	1.2	7.2	1.6	5.4	1.8	6.0	1.5	5.6	1.7	6.0	1.4	5.2
5	—	—	4.0	8.0	3.6	8.5	4.2	6.6	4.2	8.0	—	—	3.6	7.1	3.7	6.3
6	5.0	10.7	5.0	9.8	4.6	9.6	5.8	7.7	5.0	8.5	5.0	7.5	4.4	8.2	4.9	6.0
7	6.0	11.4	7.1	11.3	6.4	11.5	7.3	9.8	6.7	9.7	6.5	8.8	6.0	10.0	6.6	8.2
8	9.8	13.3	11.0	12.3	9.6	13.2	10.5	10.9	10.0	11.3	9.5	10.1	8.1	—	9.4	9.4
9	12.0	14.3	13.0	14.3	12.0	14.2	12.4	12.6	12.5	12.0	12.0	11.3	9.5	11.3	11.2	10.7
10	14.0	15.3	—	—	14.5	15.3	15.0	13.0	14.5	12.8	14.0	—	11.2	12.5	12.5	11.0

* The latencies given in Tables 1 and 2 include the time for the conduction of impulses in the afferent nerve fibres. This is, however, a constant for each preparation.

remarkably constant for each dorsal root potential. From this table we see that the latency of the dorsal root potentials increases with the distance between the root in which it is recorded and the root stimulated.

The latency of dorsal root potentials in roots caudal to the root stimulated

The slow dorsal root potential can be recorded in roots caudal to the root stimulated as well as in those cephalad to it. Thus when the 3rd dorsal root is stimulated, the slow potential changes can be detected in the 10th ipsilateral dorsal root. Table 2 shows the results obtained from another eight preparations. A sensory volley of impulses was sent into the cord through the 3rd dorsal root by stimulating the brachial nerve. The arrangement of this table is the same as that of Table 1. Here again the larger the distance between the recording root and the root stimulated, the longer the latency of the dorsal root potential.

The latency of the dorsal root potential in the root stimulated

In a previous short note [1939] the author has published a figure of the potential changes in a dorsal root when the same root was stimulated. In that figure the second phase of the diphasic spike, which indicates the



Fig. 2. The potential changes in a 10th dorsal root, when the root itself was stimulated. Recording electrodes 1 and 12 mm., stimulating electrodes 4 and 4.8 cm., from the cord. 16.0° C. (Time, 0.1 sec.)

arrival of the impulses at the electrode near to the cord, overlaps with the beginning of the dorsal root potential. So it was impossible to measure the latency of the potential changes in the root stimulated. At the end of the above experiments more attempts were made to record the dorsal root potentials in the root stimulated, and records were obtained in which the diphasic spike does not overlap with the beginning of the dorsal root potential (Fig. 2). The latency of the dorsal root potential in such records is about 4.4 msec. at room temperature

(15.5–17.8° C.). This agrees with the latencies of unitary dorsal root potentials in the frog which have been measured by Fessard & Matthews [1939].

*The direction of conduction and the latency
of the dorsal root potential*

In a fourth group of frogs the 3rd and the 10th dorsal roots on one and the same side of the spinal cord were used alternatively as root for examination and stimulation. The leading electrodes were kept in each case approximately 1 and 10 mm. from the cord, and the stimulating

A

B

Fig. 3. A, 3rd dorsal root potential when the 10th dorsal root was stimulated. B, 10th dorsal root potential when the 3rd dorsal root was stimulated. Recording electrodes 1 and 10 mm., stimulating electrodes 10 and 15 mm., from the cord. 16.8° C. (Time, 0.1 sec.)

electrodes 10 and 15 mm. from it. Thus the distance between the stimulating and recording electrodes was the same in both cases. The latency of the dorsal root potential is the same for conduction in either direction.

Fig. 3 A shows the potential changes in the 3rd dorsal root when the 10th dorsal root was stimulated. Fig. 3 B shows the 10th dorsal root potential of the same preparation when the 3rd dorsal root was stimulated. Besides the equality of the latency of these two potentials, it is interesting to notice the difference in their shape. The rising phase of the 3rd dorsal root potential is definitely slower than that of the 10th root. This difference is typical for all the records obtained in this section of experi-

ments. A small spike is often seen at the beginning of the slow dorsal root potential in the 3rd dorsal root. This spike never occurs in the 10th one.

The intensity of stimulation and the latency of the dorsal root potential

In the course of experiments reported in the first two sections above, it has also been found that the variation of the intensity of stimulus from threshold to just supramaximal caused in none of the dorsal roots any unmistakable change of the latency of their potential. A stronger stimulation gives rise only to a negativity of larger amplitude. Thus there does not appear to be any evidence of a synaptic delay varying with the size of the afferent volley preceding the start of the dorsal root potential, such as was found by Eccles & Sherrington [1931] to occur in the flexor reflex, and it accords with the hypothesis that the potentials originate presynaptically.

The latency of the dorsal root potential to the second of two centripetal volleys

Eccles & Sherrington have also found that if two centripetal volleys are sent into the spinal cord in the cat one following another within a certain time interval, the latent period of the discharge of the motor-neurones to the second volley is smaller than to the first. The largest decrease of latency occurs when the second volley is applied approximately 10 msec. after the first. Experiments to see whether this applied to the dorsal root potentials were made and it was found that the latency of the dorsal root potential to the second of two centripetal volleys is always the same as to the first centripetal volley, no matter what is the interval between these two stimuli (Fig. 4).

Strychninization, asphyxia and the latency of the dorsal root potential

If the spinal cord is subjected to an oxygen-lack environment by substituting nitrogen for air, the dorsal root potential diminishes gradually. Re-admission of oxygen at the moment when the dorsal root potential entirely vanishes can bring it back again to its normal size. Through all these stages, however, there is no unmistakable change in the time relationship of the different phases of the dorsal root potential. Nor is there any change in the latency.

By dropping 1/10,000 strychnine solution on the surface of the spinal cord, the animal can be brought into a convulsant

30 min. Although the excitability of the spinal cord becomes under these conditions enormously enhanced and the dorsal root potential prolonged. There is no shortening of its latency.

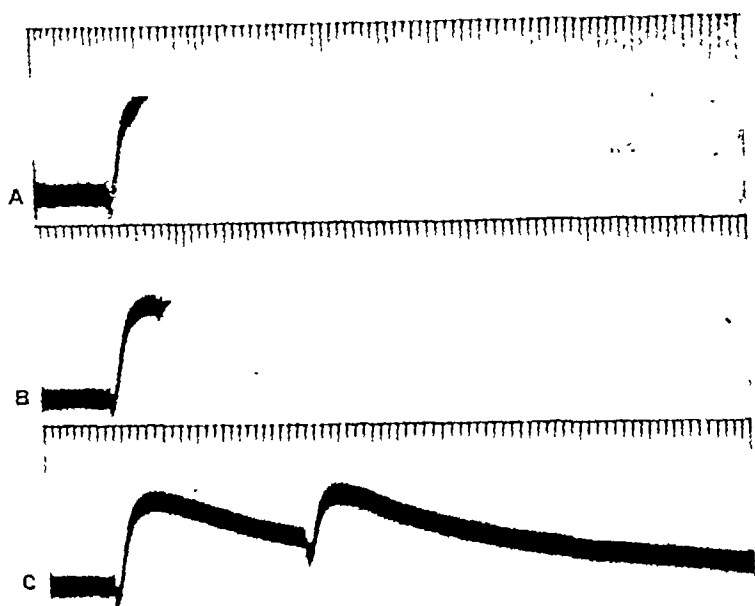


Fig. 4. Potential changes in the 8th dorsal root when two sensory volleys of impulses were sent into the cord through the 10th dorsal root with various intervals between them. Recording electrodes 1 and 10 mm., stimulating electrodes 4 and 5 cm., from the cord. 15° C. (Time, 0.01 sec.) Direct coupled amplifier.

DISCUSSION

The latency of the dorsal root potential includes the time for conduction of impulses in nerve fibres before and after their entrance into the cord and the time for the development of the slow negativity either pre- or post-synaptically. The increase of this latency when the roots in which the potential is recorded are situated farther away from the root stimulated, may be due either entirely to the increased distance for conduction or to additional synaptic delays as well. Fessard & Matthews [1939] have by recording the unitary dorsal root potentials found that the latency is about 5 msec. This agrees with the present measurement of the latency of the potential changes in a whole dorsal root when the root itself was stimulated. In Tables 1 and 2 the latency of the dorsal root potentials in the root immediately next to the root stimulated is

about 7 msec. This figure includes also the time for the conduction of the nerve impulses in the peripheral part of the nerve, which is approximately 1.5–2 msec.

Fessard & Matthews are of the opinion that the slow dorsal root potential originates pre-synaptically. However, if the dorsal root potential arises in the internuncial neurones, then this latency of *ca.* 5 msec. would be regarded as the synaptic delay. If it arises pre-synaptically we suppose that excitation of the secondary neurones takes place during the rising phase of the dorsal root potential (Dun, 1939; Barron & Matthews, 1938c).

The results in Tables 1 and 2 show that the latency of the dorsal root potential in a root most distant from the root stimulated is only about 5–7 msec. longer than that in the root nearest to it. This suggests strongly that the increase of the latency of the dorsal root potential with the distance between the root examined and the root stimulated is due entirely to the increased time for conduction and not to synaptic delays, because the increase of latency in the adjacent roots is too small to be regarded as due to an additional synaptic delay. If we postulate no additional synaptic delay in the latency of the dorsal root potentials given in Tables 1 and 2 we can calculate the rate of conduction in the spinal cord. It amounts approximately to 1–2 m./sec. and is reasonably uniform for the roots examined (see Tables 3 and 4).

TABLE 3

Frog no.

Root no.	60 L m./sec.	60 R m./sec.	61 L m./sec.	61 R m./sec.	62 L m./sec.	62 R m./sec.	63 L m./sec.	63 R m./sec.	Average m./sec.
4	—	—	—	—	—	—	1.6	—	?
5	1.5	1.2	2.0	1.2	0.8	1.4	1.4	1.3	1.4
6	1.7	—	1.8	1.3	0.9	1.2	1.6	1.5	1.4
7	2.0	1.2	1.3	1.9	1.5	1.2	2.0	2.1	1.7
8	1.7	—	1.4	1.7	1.0	1.7	1.5	1.0	1.4

TABLE 4

Frog no.

Root no.	71 m./sec.	72 m./sec.	73 m./sec.	75 m./sec.	92 m./sec.	93 m./sec.	94 m./sec.	95 m./sec.	Average m./sec.
5	—	1.8	1.8	2.2	2.2	—	1.7	2.1	1.9
6	1.2	1.3	1.4	1.8	2.0	1.8	1.2	2.1	1.6
7	1.3	1.2	1.2	1.3	1.7	1.6	1.1	1.7	1.4
8	1.5	1.7	1.4	1.6	1.9	1.8	—	1.9	1.7
9	1.6	1.5	1.5	1.5	2.1	1.8	1.5	1.8	1.7
10	1.7	—	1.6	1.8	2.2	—	1.5	1.7	1.7

Eccles & Sherrington found in the cat that if two stimuli are applied within a certain time interval, the reflex response evoked by the second centripetal volley has a smaller latent period than that by the first.

They have found also that the latent period of the flexor reflex decreases if the strength of a submaximal stimulus becomes stronger. They explained these results as being entirely due to a shortening of the synaptic delay. If the slow dorsal root potential were to arise in the internuncial neurones, then we should expect under similar conditions a shortening of the latent period of the dorsal root potentials. The results of the present experiments show that the latency of the dorsal root potential cannot be reduced either by increasing the stimulus strength or by a previous sensory volley applied at any time interval. They favoured the view that the dorsal root potentials arise at the terminations of the primary dorsal root fibres themselves and not at the internuncial neurones.

The dorsal root potential at a distant root may result from the arrival of impulses which are conducted slowly in the cord. A slow conduction in the grey matter, however, might occur, but this is dismissed on the evidence given below.

According to the theory of Barron & Matthews [1938*a*], the active depolarization of one nerve termination may cause the passive depolarization of another termination. The spread of the dorsal root potential inside the cord might, therefore, be carried out by the process of induction from termination to termination. In this case there might be a slowly conducted process travelling in the grey matter of the spinal cord comparable to that seen in the grey matter of the cortex [Adrian, 1936]. The results of the present experiments show that the rate of conduction of the dorsal root potentials in the spinal cord is about 1-2 m./sec. It is known that the intracordal parts of the primary dorsal root fibres have many collaterals and become thinner and thinner as they go farther up or down the spinal cord, and thus the conduction rate of the sensory impulses in them might be reduced to 1-2 m./sec. By alternative stimulation and examination of the 3rd and the 10th dorsal roots we have seen that the rate of conduction in both directions is the same; suggesting that no synaptic conduction is involved. The difference in the shape of these two potentials is however considerable. This may be due to different local conditions prevailing in the neighbourhood of the entrance of the two different roots, but it is equally possible that the two dorsal root potentials are the result of sensory impulses which are conducted in the spinal cord in different groups of primary dorsal root fibres. In the latter case the difference in the shape of the potentials would result from differences in the degree of dispersion of the sensory impulses.

PART II

The conduction of the dorsal root potentials

If the dorsal root potentials were conducted by some process of induction from termination to termination, then the grey matter would be the conducting part of the spinal cord. If, on the other hand, the production of the dorsal root potentials in the distant roots depended mainly on conduction of sensory impulses in the primary dorsal root fibres, then the dorsal column would be the conducting tissue. In the following series of experiments the effect of various spinal cord lesions on the production of the dorsal root potentials in the distant roots has been investigated, and it has been found that the dorsal column is the main structure in the spinal cord responsible for the conduction of those processes which produce the dorsal root potentials.

Dorsal and ventral hemisection

If a dorsal hemisection is produced at the level between the 9th and the 10th segments, all the dorsal root potentials in the roots beyond the lesion are eliminated. The stimulation of the 10th dorsal root causes only a negativity in the root itself. A stimulation of the 9th dorsal root or any dorsal root cephalad to it gives rise to potential changes in all the roots except the 10th one. If a dorsal hemisection of the spinal cord is produced just below the 3rd dorsal root, the stimulation of it is followed by no potential changes in any of the lower caudal dorsal roots. The hemisection of the dorsal half of the spinal cord blocks the spread of the dorsal root potentials in both directions. A ventral hemisection, on the other hand, at the corresponding levels of the spinal cord has no detectable influence on the production of the potential changes in roots beyond this lesion, even when it extends far above the level of the central canal occupying nearly four-fifths of the cross-section of the whole spinal cord (Fig. 5).

The relation between the dorsal and ventral root potentials

After seeing that the dorsal hemisection alone stops the production of the dorsal root potentials in the distant dorsal roots, the question arises whether the ventral hemisection has any effect on the production of ventral root potentials in the ventral roots beyond it. It has been found that the presence of the ventral root potentials depends largely on the presence of the dorsal root potentials in the same level of the spinal cord. If a dorsal hemisection is produced at the level between the 9th and the 10th segments of the spinal cord, the stimulation of the

10th dorsal root fails not only to produce the potential changes in the 9th dorsal root but in the 9th ventral root as well. If a ventral instead of a dorsal hemisection is produced at the same level, the 9th ventral root potential can be recorded without any apparent diminution in amplitude, when the 10th dorsal root is stimulated.

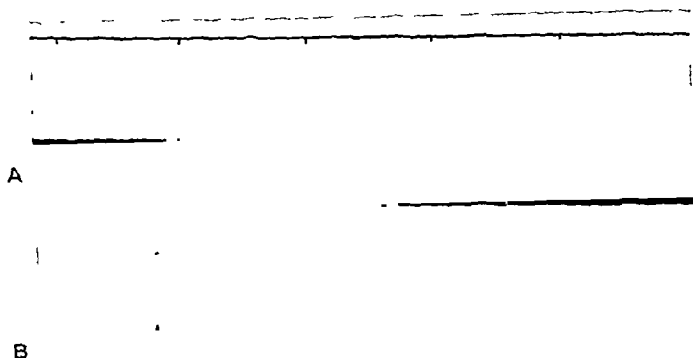


Fig. 5. A, frog C 12. After *dorsal* hemisection at the level between the 9th and 10th segments, no potential changes can be detected in the 8th dorsal root when the ipsilateral 10th dorsal root is stimulated. B, frog C 14. After *ventral* hemisection at the same level the 8th dorsal root shows potential changes as before when the 10th dorsal root is stimulated. Recording electrodes 0.8 and 15 mm., stimulating electrodes 4 and 5 cm., from the cord. 15.0° C. (Time, 0.08 sec.)

Lateral and bilateral hemisection

In a number of preparations hemisection was produced on one side of the spinal cord at the level between the 9th and the 10th segments. The lesion extended in each case just beyond the middle line of the spinal cord. The stimulation of the 10th dorsal root on the side of the lesion caused in the ipsilateral 8th and 9th dorsal roots no potential changes whatever. On the contralateral side potential changes can be recorded in the 10th, 9th and 8th dorsal roots as in the normal preparation, but no potential changes can be detected in root more distant than that. The ipsilateral 8th and 9th dorsal roots gave potentials when the contralateral 10th dorsal root was stimulated. The amplitude of these potential changes was only slightly reduced in comparison with that recorded just before the operation.

The effect of two hemisections one on each side of the spinal cord 2 mm. apart is similar to that of a dorsal hemisection. The stimulation of one root on one side of the bilateral hemisections can cause no potential changes in roots on the other side of the lesions.

The dorsal column and the potential changes in the distant roots

After it seemed fairly clear that it is the sensory impulses conducted in the dorsal column which give rise to the potential changes in the distant roots, attempts were made to isolate the dorsal column at the level between the 9th and the 10th segments. This was done by first making a ventral hemisection and then destroying the remaining grey matter with a needle. This method proved to be quite easy and efficient, if carried out under a microscope. Several preparations which showed large 8th dorsal root potentials when the 10th dorsal root was stimulated, proved later to have nothing but the dorsal column intact at the level of the lesion.

DISCUSSION

Our results suggest that the dorsal root potential at any level is evoked by impulses that travel slowly in the spinal cord. The potential changes on the contralateral side might be evoked by impulses travelling in collaterals of the primary dorsal root fibres which cross over to the other side of the spinal cord through the dorsal commissure [Gaupp, 1899]. Slow conduction of activity from neurone to neurone observed by Adrian [1936] in the cortex of mammals does not seem to be responsible for the slow conduction we observed in the frog spinal cord because it occurs when only the dorsal columns are intact. Many fibres of the dorsal columns of the frog, e.g. those going to the medulla, conduct rapidly (18–25 m./sec., Matthews' personal communication). These fibres cannot be responsible for the conduction of the impulses which evoke the dorsal root potentials. There are many very fine fibres in the dorsal column of the frog, and it appears likely that these carry the impulses that evoke the dorsal root potentials in distant parts of the cord. Measurements of latency of dorsal root potentials evoked by stimulation of the sciatic nerve and those by stimulation of a root show, however, that the peripheral parts of the fibres responsible for evoking the dorsal root potential conduct rapidly and belong to the A group of Gasser and Erlanger.

Eccles & Sherrington [1931] defined the 'synaptic delay' as 'the time necessary to build up c.e.s. to threshold value' or 'the interval between the incidence of the first impulse on a motor neurone and the setting up of a reflex discharge'. The length of the synaptic delay is determined by the dispersion of the impulses arriving at the motor neurone and by the c.e.s. already present there. It varies with the stimulus strength as well as the interval between the conditioning and the testing sensory volleys. These variations have not been found in the latency of the dorsal root potentials.

If we suppose that the beginning of the slow potential change indicates the beginning of C.E.S. and arises pre-synaptically, the above-mentioned difficulties do not arise. And the reflex latencies (*ca.* 20 msec.) observed by Bremer & Kleyntjens [1937] and Kleyntjens [1937] agree with the hypothesis that reflex excitation of secondary neurones occurs during the rising phase of the dorsal root potential, as in the case of re-excitation of dorsal root fibres when the spinal cord has been cooled [Barron & Matthews, 1938*b*; Dun, 1939].

The potential changes observed indicate physico-chemical changes occurring in the cord. The hypothesis that excitation occurs on the rising phase of the dorsal root potential is not incompatible with transmission being mediated by some specific ion such as that of acetylcholine. Presumably, however, both the electrical change and constitution of the ion are significant and, as the two are inseparable, discussion of which is responsible for excitation is perhaps not at present of great profit.

SUMMARY

1. When a volley of sensory impulses is sent into the spinal cord, potential changes can be detected in practically every dorsal root on the ipsilateral side and also in some roots on the contralateral side.

2. The latency of the dorsal root potential in the root stimulated is *ca.* 4.4 msec.

3. The latency of the other dorsal root potentials increases with the distance between the root in which it is recorded and the root stimulated.

4. This increase of latency is thought to be due to increased distance of conduction and not to synaptic delays.

5. Variation in the intensity of stimulation does not change the latency of the dorsal root potential. Nor is it influenced by a previous sensory volley, no matter what is the interval between these two stimuli.

6. Asphyxia diminishes the dorsal root potential, but does not lengthen its latency. Strychninization increases the dorsal root potential but does not shorten its latency.

7. If the 3rd and the 10th dorsal roots on the same side are used alternatively as recording and stimulating roots, the latency of these two potentials is the same, but the difference in their shape is considerable.

8. The production of the dorsal root potentials in the roots other than that stimulated depends on the intactness of the dorsal column, which conducts the sensory impulses to them. The dorsal root potentials on the contralateral side are thought to be evoked by impulses travelling in the collaterals which form the dorsal commissure.

9. The rate of conduction of impulses responsible for the production of the dorsal root potentials is of the order 1-2 m./sec. in the cordal part of the fibres.

10. The view that the dorsal root potentials arise presynaptically is strengthened by the above results.

11. The beginning of the slow dorsal root potential is supposed to indicate the beginning of c.e.s. and the excitation of secondary neurones is supposed to occur during the rising phase of the dorsal root potential. This hypothesis is shown to be in agreement with the measurements of the reflex latencies by Bremer & Kleyntjens and Kleyntjens.

12. The above hypothesis is not incompatible with transmission being mediated by some specific ion, e.g. that of acetylcholine.

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STRENGTH AND SIZE OF BONE IN RELATION TO CALCIUM INTAKE

BY G. H. BELL, D. P. CUTHBERTSON AND J. ORR

From the Institute of Physiology, and the Department of Engineering, University of Glasgow

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BONE serves three main functions in the body: (1) it gives rigidity to the skeletal framework thus supporting the soft tissues and facilitating movement; (2) it acts as a readily available source of minerals; and (3) it has a haematopoietic action. We are here concerned with the first two functions only.

Fairbanks & Mitchell [1936] have raised the question as to whether a very rapid rate of bone calcification is a benefit. They express the view that benefits accruing from increased rates of calcification of the skeleton only occur up to a point probably considerably below complete saturation. On the other hand, they hold that 'until the minimum percentage saturation of the stores compatible with maximum physiological performance has been determined it would seem to be the wiser course (certainly the safer course) to consider complete saturation of the store as the ideal condition...'. It is obvious that something more than mere chemical observations are required to supply such information.

In recent work on bony growth the quality of the bone has been judged chiefly on the basis of chemical analysis, but this seems, to the present writers, to disregard the essential function of bone as a supporting tissue. A bone must be strong enough to bear the weight of the body, to resist the pull of the muscles, and to withstand the strains and stresses of an active life. A certain amount of work has been done in estimating the strength of human bones, and this was reviewed by Disse [1896]; several estimates have also been made on the strength of the bones of swine and cattle [Becker & Neal, 1931; Becker, Neal & Shealy, 1934; Maj, 1938]. Lindsay & Howes [1931] and Lindsay [1934] have described strength tests on normal and healing fractured fibulae of rats.

None of these reports gives any information about the quality of bony material, nor is advantage taken of the methods of measuring strength of materials well known to engineers.

The experiments here to be described were made with a view to correlating functional and chemical observations. In particular, we were anxious to know how the quality of the body material was affected by a much reduced and a much increased Ca intake.

EXPERIMENTAL PROCEDURES

(a) Biological

Ninety-six recently weaned male albino rats were used in the four experiments and were fed on Rowett Institute stock diet [Thomson, 1936] until they were about 60 g. in weight. In each experiment the animals were divided up into groups of four or five; one representative group was sacrificed at the beginning of each experiment and the Ca content determined after weighing the degutted carcasses. The remaining groups were placed on diets of constant protein, carbohydrate, fat and vitamin content but of varying Ca content (see Table 1) over a period of 56 days. Each animal was housed in a separate galvanized wire cage with feeding attachments of the Hopkins pattern. The food was given *ad lib.* and was made up as follows:

	%
Dried egg albumin	5.0
Dried egg yolk	10.0
Potato starch	15.0
Rice starch	37.0
Cane sugar	10.0
Butter fat	10.0
Dried yeast	5.0
Cod liver oil (Seven Seas Ltd. standard potency)	3 drops daily
de Loureiro's salt mixture excluding CaHPO_4 [de Loureiro, 1931]	3.4
CaHPO_4 and BaSO_4 in various ratios making together	4.5
	<hr/> 99.9

By varying the relative amounts of CaHPO_4 and BaSO_4 it was possible to produce a range of diets with from 0.075 to 1.390 g. Ca %, the Ca contents being checked by analysis. Lower values could not be obtained because of the Ca associated chiefly with the dried egg. The basic diet, less the CaHPO_4 , contained 0.066 % Ca and 0.223 g. % P; the Ca:P ratio of the total diets ranged from 0.33 to 1.11.

This system of altering the Ca in the food without disturbing the rest of the diet is similar to that used by Fairbanks & Mitchell [1936] and has of course the defect inherent in all such experiments of causing a variation in the Ca:P ratio. It is obvious

proportionate restriction in P when the Ca is reduced would impose effects on soft tissue growth which would complicate the issue, and further, any rachitogenic action, due to the abnormal Ca:P ratio of our diets, was presumably prevented by giving adequate amounts of vitamin D.

The first twenty animals used in Exp. I came from one dealer. The remaining seventy-six used in Exps. II-IV came from another dealer. Exp. I cannot, therefore, be directly compared with Exps. II-IV, although the results vary in the same direction. Exps. II-IV can be directly compared, and the results in any one of these three experiments can be more closely scrutinized since all the animals were of the same stock and were fed under the same climatic conditions.

The animals were weighed at weekly intervals for the 8 weeks of the experiment. During this time the total food intake was noted so that the total consumption of Ca could be calculated. Owing to spillage, inevitable in such experiments, the values are probably slightly in excess of the actual amount eaten.

At the end of the experimental period the animals were killed and then weighed, the body length measured, and after degutting they were weighed again. (This method of discounting the Ca not actually within the animal's tissues by removing the entire alimentary canal was preferred to the rather unphysiological procedure of 24 hr. starvation before killing, a process which results in a very great loss of weight with presumably a call on the reserves of Ca among other materials.) The femora were dissected out, cleaned of muscle and tendon, then skia-graphed; finally, they were weighed and measured and used for the mechanical tests. Their Ca content was estimated by analysis of the residual fragments from these tests. The Ca content of the rest of the carcass was also determined so that it was possible to estimate the retention of Ca in the whole animal (less alimentary canal) over 56 days, due allowance being made for the Ca of the femora. The percentage of Ca in the degutted rat is expressed on the basis of the degutted weight. Since drying occurs very quickly in these bones it was impossible to weigh, measure, and X-ray the bones and then carry out the mechanical tests with the bone in its fresh, moist state. The bones were, therefore, allowed to dry at room temperature for several days prior to being weighed, no further loss of weight being likely to occur in the process of mounting the bones for the mechanical tests. The fragments were of comparable dryness, i.e. in equilibrium with the air, and the weights given in Table 1 may be regarded as those of air-dried bones. It is

obvious that more drastic drying might have altered the mechanical properties of the femora. Bachmann, Haldi, Wynn & Ensor [1940] found that the percentage of Ca in the femora of male rats about the same age as ours which were fed on rations varying from 0.4 to 1.0 % Ca over a similar period of time varied from 22.3 to 23.3 % of the dry weight. This is in general agreement with the values given in our table.

The Ca was determined in the food, carcasses, and bones by appropriate modifications of Shohl & Pedley's method [1922].

(b) *Mechanical*

In our choice of tests we have been guided by the experience already available to engineers concerned with the determination of the strength of materials. These tests usually consist in determining the load necessary to fracture a test sample. When selecting a mechanical test for a bone it is important to simulate as closely as possible the straining actions taking place in the living animal. There are four types of straining action possible—axial compression, axial tension, bending and twisting. If a bone were tested by a crushing load applied axially at the ends failure would probably take place in the bone immediately under the loading points. Even if the bone were protected so that the fracture occurred in the shaft, it is doubtful if the breaking load observed would be a reliable index of strength. The chief difficulty with this type of test is to ensure that the load will be applied directly along the long axis of the bone—this is especially difficult when the specimens are small but is highly important, since a small eccentricity of loading may halve the strength of the bone. It is obvious from the shape of the femur that, since the line joining the head and the condyles lies mostly outside the shaft, true axial loading never occurs in the living animal. Eccentric loads produce bending in a columnar structure, and this is more severe in its effect, than direct compression; it is, therefore, much more important to determine bending strength. Further, if the example of the adductor magnus muscle is considered it will be realized that muscles may produce quite severe bending actions. It will be shown later that the femur is much stronger than is necessary from considerations of axial compression alone. Bones are seldom called upon to resist axial tension; even in the highly abnormal procedure of reducing a dislocation the strain is borne chiefly by ligaments and muscles. We have confined our attention, therefore, to tests of twisting and bending which are the actions to which bone is normally subject. The actions are brought about by muscular pull, by the weight of the body and by accidental violence.

Bone is usually broken by impact, i.e. sudden application of a force, either when an external object strikes the limb or when the animal strikes the ground after a leap or fall. A test involving impact loading, however, is very difficult to evaluate with any accuracy especially when the specimens are small; hence gradual application of the load during a test until the bone breaks is a more satisfactory procedure. In testing engineering materials it is customary to place greater reliance on tests involving static loading than on impact tests even where the member tested, e.g. a piston rod, has to withstand impact.

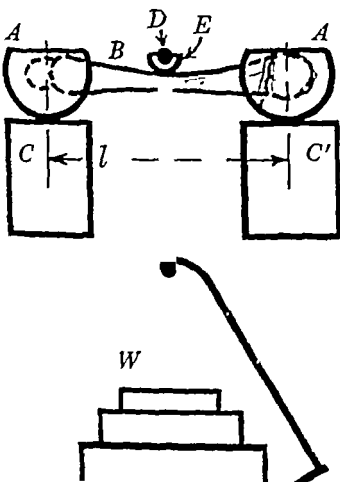


Fig. 1.

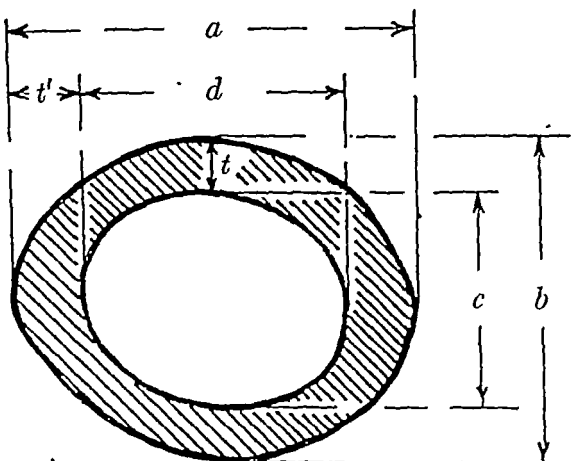


Fig. 2.

Fig. 1. Apparatus for determination of bending strength. *B*, bone; *A*, semicylindrical ends cast on to bone; *C*, *C'*, supports; *D*, wire with red fibre lining *E*; *W*, load.

Fig. 2. Diagrammatic section through the midpoint of the shaft of the femur to indicate where the measurements were made.

Bending test. Each right femur, *B* in Fig. 1, had its ends cast into short semi-cylindrical pieces, *A*, of a hard resinous substance (a cement composed of equal parts of plaster of Paris and colophony resin which, when heated up with a little tallow, forms a homogeneous mixture). This process prevented the bone from crushing at the line of contact with the supports, *C* and *C'*, provided an accurate measurement of the span, *l*, and also prevented axial rotation. For the sake of uniformity care was taken with casting on the ends to place the bone with the major axis of the central section horizontal, i.e. parallel to the axis of the cylindrical end pieces. This was accomplished by laying the bone in a little moulding machine and pouring the molten cement over and around

obvious that more drastic drying might have altered the mechanical properties of the femora. Bachmann, Haldi, Wynn & Ensor [1940] found that the percentage of Ca in the femora of male rats about the same age as ours which were fed on rations varying from 0.4 to 1.0 % Ca over a similar period of time varied from 22.3 to 23.3 % of the dry weight. This is in general agreement with the values given in our table.

The Ca was determined in the food, carcasses, and bones by appropriate modifications of Shohl & Pedley's method [1922].

(b) *Mechanical*

In our choice of tests we have been guided by the experience already available to engineers concerned with the determination of the strength of materials. These tests usually consist in determining the load necessary to fracture a test sample. When selecting a mechanical test for a bone it is important to simulate as closely as possible the straining actions taking place in the living animal. There are four types of straining action possible—axial compression, axial tension, bending and twisting. If a bone were tested by a crushing load applied axially at the ends failure would probably take place in the bone immediately under the loading points. Even if the bone were protected so that the fracture occurred in the shaft, it is doubtful if the breaking load observed would be a reliable index of strength. The chief difficulty with this type of test is to ensure that the load will be applied directly along the long axis of the bone—this is especially difficult when the specimens are small but is highly important, since a small eccentricity of loading may halve the strength of the bone. It is obvious from the shape of the femur that, since the line joining the head and the condyles lies mostly outside the shaft, true axial loading never occurs in the living animal. Eccentric loads produce bending in a columnar structure, and this is more severe in its effect, than direct compression; it is, therefore, much more important to determine bending strength. Further, if the example of the adductor magnus muscle is considered it will be realized that muscles may produce quite severe bending actions. It will be shown later that the femur is much stronger than is necessary from considerations of axial compression alone. Bones are seldom called upon to resist axial tension; even in the highly abnormal procedure of reducing a dislocation the strain is borne chiefly by ligaments and muscles. We have confined our attention, therefore, to tests of twisting and bending which are the actions to which bone is normally subject. The actions are brought about by muscular pull, by the weight of the body and by accidental violence.

along the shaft for some distance; the thickness of the shell of the femur was measured at several places on the fracture site.

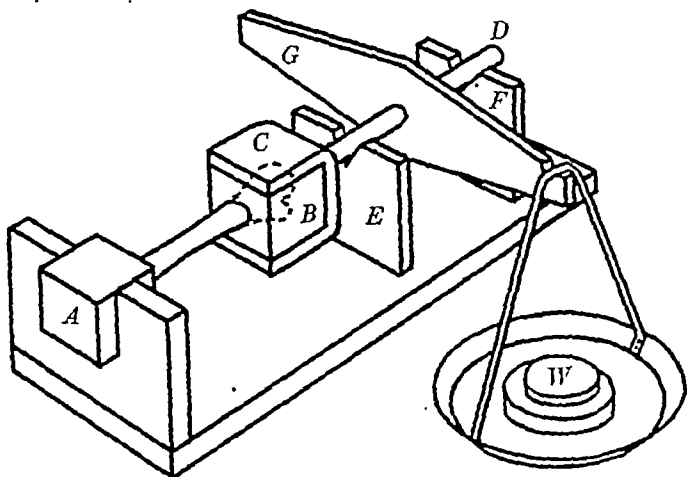


Fig. 4. Machine for measuring twisting strength. *A* and *B*, brass cubes cemented on to the ends of the femur; *C*, grip; *D*, spindle; *E* and *F*, supports; *G*, lever; *W*, load.

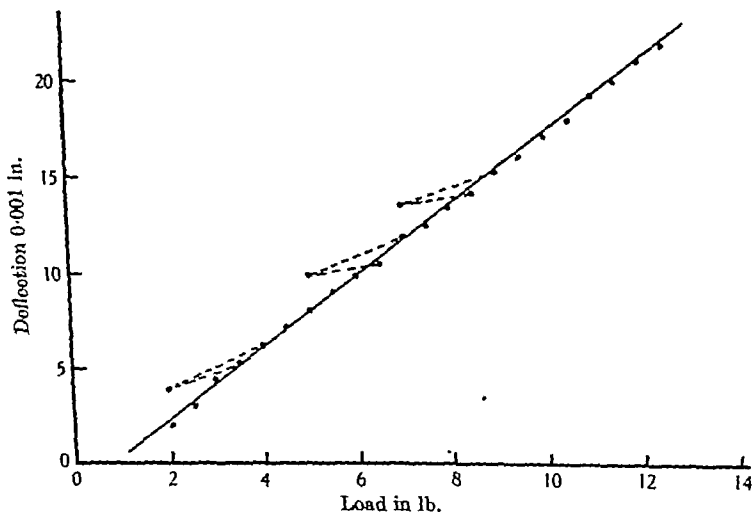


Fig. 5. Displacement of the midpoint of the shaft from its unloaded position during the bending test. The bone fractured under the conditions indicated approximately by the end of the line.

Deflexion test on bending. This test consisted in measuring the displacement of the midpoint of the shaft by means of a dial gauge resting lightly on *D* of Fig. 1 while adding weights during the bending test.

the ends. The span, l , was measured by vernier calipers, and the external dimensions, a and b (see Fig. 2), at the central section by a micrometer reading to $1/10,000$ in. The loading W was applied by adding weights to the pan which was hung by a steel wire D , in Fig. 1, looped round the centre of the shaft. A ring of red fibre E reduced the danger of local crushing of the bone material. The load was increased by small steps until the bone broke. The actual breaking load was taken to lie between the greatest weight supported and the final weight applied; the error involved is small because the steps were a small fraction of the total weight including the pan. The thicknesses, t and t' of Fig. 2, were measured by the micrometer at the fracture site.

The weakest section of the bone is at the midpoint of the shaft where the dimensions are smallest; the bending moment is also greatest there, having a value $W/2 \times l/2 = \frac{1}{4}WL$ as shown by Fig. 3. From this test we can calculate the bending moment M at the midpoint of the bone where it breaks. If the span l had been maintained at a constant value throughout the experiments the breaking load W would have been an index of the bending strength of the shaft of the bone. In practice it was found more convenient to allow the span to vary a little and therefore the greatest bending moment, $\frac{1}{4}WL$, is used as the index of the strength of a bone.

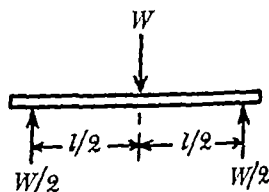


Fig. 3. To illustrate bending moment.

Twisting test. In this test hollow brass cubes were cast on the ends of the left femora by using the cement previously described. Alignment of the cubes was obtained by using a jig during the casting on process. The external dimensions, a and b , of the central section of the shaft were measured. The bone was then fitted into the machine shown in Fig. 4; end A was fixed, end B received the twisting action through the movable part comprising grip C , spindle D , and lever G . Loading W was applied gradually until the bone broke. Friction between the spindle and the supports E and F was obviated by oiling them. End B rotated through about 30° before the break occurred; the position of the lever G on its spindle was so adjusted that the bone broke when the lever was nearly horizontal. The strength of the bone in twisting is measured by the product of the breaking weight and the leverage Wl . As this product, the twisting moment or T , is constant along the bone shaft, it will cause fracture at the weakest section, usually near the centre of the shaft. In this test the line of fracture often ran obliquely or spirally

where the dimensions are for the central or fracture section as in Fig. 2, and where t_m is the minimum thickness of the wall of the femur. Formulae (3) and (4) are based on the assumptions that the section contours are elliptical and that the material is perfectly elastic. The results indicate that there is good agreement between the simple and the more complicated formulae. Formulae (3) and (4) have additional interest since they provide absolute values for the breaking stresses, s_1 and s_2 .

These formulae may be found in any standard work on strength of materials [e.g. Case, 1925].

The drying in air produced no significant alteration in the strength of the bones. Two pairs of femora from two animals were compared. Immediately after killing, one femur was removed and set up as quickly as possible for the bending test. The other bone was removed and was kept for 2 weeks in one case, and 10 months in the other. The differences in bending moments of the fresh bone and of the air-dried bone were in both cases less than 2 %.

RESULTS

The complete data of the experiments are given in Table 1 and are graphically described in Figs. 7-9. The animals of Exp. I ate considerably more food than the other animals (Exps. II-IV) which were of a different stock. The former had heavier femora and contained more Ca than the other animals fed on similar diets. The data of Exp. I are included in Table 1 but not in Figs. 7-9; for although they demonstrate the same characteristics of structural change with increasing dietary Ca they cannot be fitted into the curves of the other experiments and will not be included in the discussion unless specifically referred to. Even though the animals of Exps. II-IV are of the same stock the data show minor variations from experiment to experiment presumably for the reasons mentioned earlier.

The percentage of Ca in the diet ranges from 0.075 to 1.390 g./100 g. dry food. This is well below and considerably above the Ca content of standard diets for the growing rat. The experimental data of Orr, Thomson & Garry [1935] and of Gaunt, Irving & Thomson [1938] show that a diet containing 0.284 g. Ca/100 g. dry matter is adequate for growth and reproduction in the rat. The total food intake remains remarkably constant in spite of the marked variation in the Ca content of the food; such small divergencies as occur do not seem to be related to the Ca content of the food. Fig. 6 shows that the relation of percentage of Ca in the diet to the total Ca intake is a straight line one. It will be seen that in Exps. III and IV the percentage retention of Ca

We thought it possible that some bones might yield considerably at the higher loads; this phenomenon occurs with ductile materials like steel. The graph of the relation between the deflexion and the load in our case is a straight line up to the moment of fracture (see Fig. 5); hence bone material is elastic up to fracture. The dotted lines indicate the result of reducing the load a little to a previously observed value: the deflexion was then somewhat greater than was observed originally at this loading. On increasing the load once more the deflexions fell again on the original straight line. These departures from the straight line must be due to crushing of the end mouldings on the steel supports. Since no differences were detected between the bones by this test it was carried out in a few instances only.

Breaking stress. The strength of a bone shaft depends on the shape and dimensions of the central section, and on the breaking stress of the bone substance. The latter is defined by considering the part force acting on a small area of the section; the ratio part force/small area is the stress at this point. When with increasing loads the stress on any one point on the section attains a certain value fracture occurs there and spreads immediately throughout the section. This value is the breaking stress which is a measure of the strength quality of the bone substance. From our measurements we calculated the breaking stress to determine if lack of Ca in the diet affected the quality of the bone laid down under these circumstances.

The formulae used for calculating breaking stress are

$$M = s_1 (abt), \quad (1)$$

and
$$T = s_2 (abt), \quad (2)$$

where M = bending and T = twisting moments at fracture, s_1 , s_2 = breaking stress in bending and twisting respectively, and a , b , t are the dimensions of the central or fracture section as indicated in Fig. 2. These formulae do not give absolute values for the breaking stress but give values which are comparable among themselves. They assume that the section profiles of the various specimens are similar in shape with proportional dimensions. Since the section profiles of the specimens were not exactly similar an error of an unknown amount is introduced. Two additional formulae derived on a somewhat different basis were used as a check. These are

$$M = s_1 \left(\frac{\pi}{32} \frac{ab^3 - dc^3}{b} \right), \quad (3)$$

and
$$T = s_2 \frac{1}{2} \pi (a - t') (b - t) t_m, \quad (4)$$

at the lowest levels of intake was somewhat less than at the second lowest level. With increasing intake there is a diminution in the percentage retention of Ca, but the absolute amount of Ca retained increases up to nearly 1.5 g. over 56 days when the food contains 0.36 g. Ca/100 g.; increase of dietary Ca beyond this point produces very little increase in retention. There is, therefore, little doubt that an intake above this amount is a *luxus consumption*.

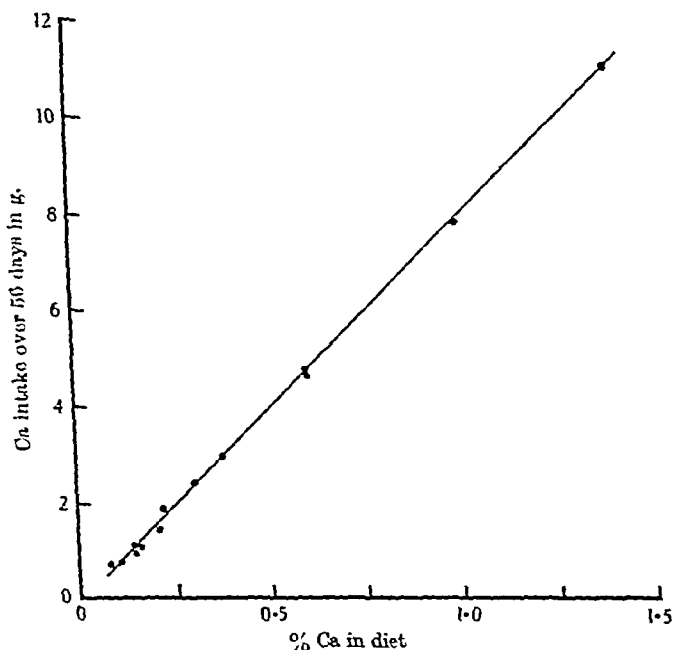


Fig. 6. The relation between the percentage of Ca in the diet and the total Ca intake.

The percentage of Ca in the animal showed the same range of values as found by Fairbanks & Mitchell [1936]—their empty weight is presumably the same as our degutted weight. The figures relating to Ca content of the degutted carcasses show interesting physiological adjustments. When the percentage of Ca in the diet was varied from 0.075 to 0.36—a ratio of 1:5—the amount of Ca in the body increased from about 0.9 to 2.0 g.—a ratio of 1:2; under the same conditions the Ca content of the femur increased in the ratio 1:2. The similarity of these ratios suggests that the Ca was distributed evenly over the skeleton on both high and low diets. The quantity of Ca in the animals at the beginning of the experiment made it necessary for the Ca retention to be trebled

Experiment	No.	Ca in femur (air dried)	Measurements in 0.001 in. of the femoral shaft (Fig. 2)					Bending moment M in. lb.	Twisting moment T in. lb.	Breaking stress in bending and twisting calculated from formulae (1), (2), (3) and (4)			
			a	b	t	r	Width of marrow cavity $c = b - 2t$			(1) lb./in. ² × 2100	(2) lb./in. ² × 990	(3) lb./in. ²	(4) lb./in. ²
I, 7. vii. 38- 1. ix. 38	4	967	0.269	2.001	59	115	19.1	0.430	1.541	0.909	32.1	0.517	0.116
	4	910	0.527	4.798	60	155	19.1		1.674	0.941	19.0	0.560	0.133
	4	911	0.921	8.390	50	166	19.8		1.759	1.118	15.0	0.553	0.126
	4	898	1.303	11.701	59	161	19.4						
II, 28. x. 38- 23. xii. 38	5	730	0.141	1.031	59	162	20.5	0.473	0.765	0.619	74.2	0.290	0.067
	4	840	0.218	1.852	58	181	21.5		1.185	0.753	64.0	0.374	0.087
	4	730	0.301	2.195	60	174	20.7		1.355	0.850	61.7	0.389	0.097
	5	811	0.365	2.960	60	174	21.1		1.469	0.905	49.6	0.407	0.102
III, 11. ii. 39- 8. iv. 39	5	748	0.131	0.980	63	146	20.2	0.457	0.771	0.674	78.7	0.277	0.066
	5	718	0.144	1.034	64	141	20.0		0.876	0.733	84.7	0.289	0.066
	4	686	0.203	1.393	67	144	19.9		1.150	0.878	82.5	0.327	0.083
	5	788	0.596	4.696	64	167	20.9		1.544	0.972	32.9	0.407	0.095
IV, 15. v. 39- 10. vii. 39	5	875	0.075	0.656	62	141	20.5	0.486	0.466	0.501	71.1	0.256	0.045
	5	772	0.100	0.772	62	146	20.3		0.642	0.686	83.2	0.267	0.057
	5	771	0.600	4.626	62	159	21.1		1.372	0.929	29.7	0.382	0.090
	5	775	1.060	7.750	62	159	21.1		1.450	0.978	18.7	0.406	0.093
IV, 17. v.	4	782	1.390	10.870	62	161	21.1		1.477	0.981	13.6	0.404	0.093
I, 7. vii. 38- 1. ix. 38	21.4	146.5	120.0	15.3	16.3	—	89.4	4.64	2.40	17.8	9.08	—	—
	22.4	146.3	118.2	15.9	16.3	—	86.4	5.34	2.08	19.8	7.48	—	—
	23.8	143.8	118.6	15.3	16.3	—	88.0	5.15	2.63	19.6	8.61	—	—
	22.8	147.5	118.6	15.5	16.3	—	87.6	5.76	2.12	18.3	6.81	—	—
II, 28. x. 38- 23. xii. 38	23.0	124.5	102.0	12.6	16.3	16.3	77.4	2.99	1.52	17.75	10.24	33.440	8.500
	23.2	131.8	103.8	17.5	24.2	24.2	68.8	3.12	2.24	15.95	8.63	32.000	9.700
	25.0	128.1	96.1	18.1	21.5	21.5	62.9	3.82	2.29	16.87	10.07	38.580	10.200
	25.0	132.1	105.5	18.6	23.5	23.5	68.3	4.16	2.43	15.30	9.87	33.820	9.700
III, 11. ii. 39- 8. iv. 39	23.7	119.9	99.0	13.1	18.7	18.7	72.8	2.43	1.75	15.78	11.23	29.080	10.800
	22.8	117.5	97.6	13.6	15.9	15.9	70.3	2.68	1.79	16.82	11.79	33.370	10.800
	25.2	115.5	98.8	16.0	20.3	20.3	66.9	3.35	2.00	17.30	12.56	36.900	11.700
	23.2	134.3	101.1	18.3	25.1	25.1	64.5	4.12	2.22	14.97	9.83	33.210	9.400
IV, 17. v.	17.5	126.6	104.8	9.8	12.7	12.7	85.2	2.24	1.07	17.05	8.67	29.770	7.000

Weight of femur

It will be seen from Fig. 7 that the total Ca content of the femur, the thickness of the femoral shell and the weight of the femur are very closely correlated. This is due to the fact that the bone is of practically constant

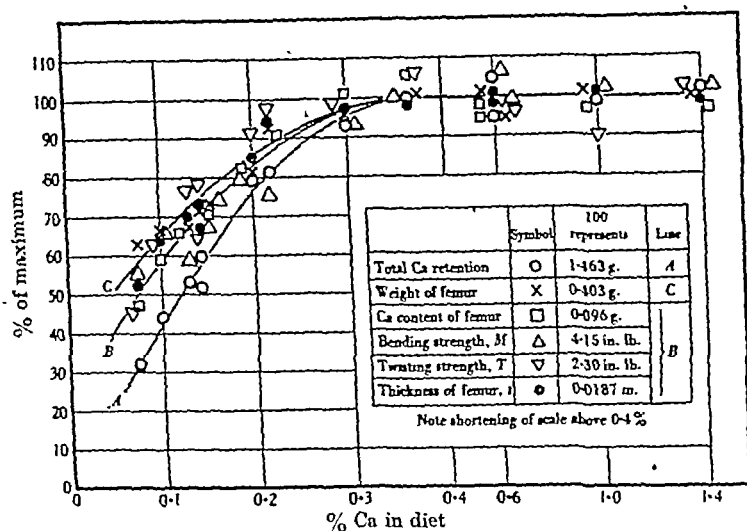


Fig. 7. Various findings, as shown in the panel in the graph, expressed as a percentage of their maximum value plotted against the percentage of Ca in the diet. (Note shortening of the scale above 0.4 %.)

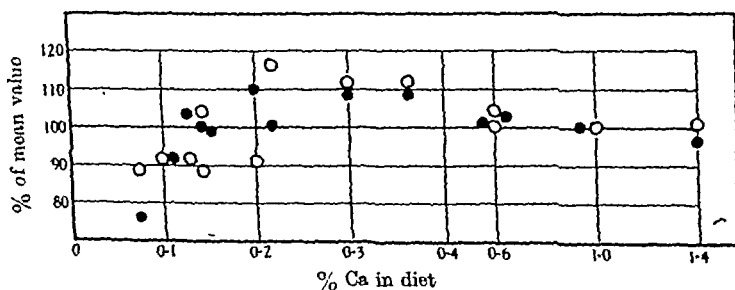


Fig. 8. Increase in weight of the whole animal and percentage of Ca in the whole femur, both expressed as a percentage of the mean value, plotted against percentage of Ca in the diet. Increase in weight, ○; 100 represents 159.2 g. Percentage Ca in whole femur, ●; 100 represents 23.0 %.

chemical composition in spite of dietary variations, with the possible exception of that formed under the poorest Ca intake (see Fig. 8). The diminution of the percentage of Ca in these circumstances is most likely due to the greater proportion of periosteal and fibrous tissue on

in order to double the actual content of Ca in the body. A further increase in dietary Ca up to 16 times the lowest produced no further change.

Body weight

The gain in body weight is given in Table 1 and Fig. 8. The average rate of growth per day over the entire period was 2.85 g. On the higher Ca intakes the gain in body weight per gram of food eaten tended to be slightly greater than on the low Ca intakes. This is quite clearly shown in the data of Exps. III and IV, but Exps. I and II do not show this tendency. It may be—although the evidence is slight—that on the higher Ca planes body tissue is formed more economically than on the lowest intakes.

Owing to the relatively constant rate of growth of our animals we could not demonstrate the relationship shown to exist by Fairbanks & Mitchell [1936] between the percentage of Ca in the carcass and the average daily gain in body weight for several weeks before killing. Nevertheless, the percentage of Ca in the carcasses of our animals fed on the higher diets was of the same order as that found by these authors.

Skeletal dimensions

Neither restriction of Ca nor *luxus* consumption of Ca affects appreciably the body length (snout to anus) or the femoral length (head to condyles in the long axis of the bone). There is a slight tendency for the greater transverse diameter (a of Fig. 2) of the middle of the shaft of the femur to be greatest in the animals fed on the highest Ca diets. The smaller diameter, b , on the other hand, remains remarkably constant in all animals. On the whole the external dimensions of the femur are not altered by varying the Ca intake over the range used in these experiments. The thickness of the femoral shell, t , increases with rise in the percentage of Ca in the diet up to about 0.36 %; thereafter it remains practically constant (● in Fig. 7). It will be clearly seen from the column of Table 1 headed 'Width of marrow cavity' that the increase in thickness is achieved at the expense of the marrow cavity. The thickness of the femoral wall measured across the lateral edges of the shaft (t' of Fig. 2) is very variable indeed, but in spite of this the same general tendency is seen. There was no cancellous tissue in the middle of the femoral shaft of these animals.

it has to be noted that formula (3) includes t' . The quality of the bone as indicated by the bending tests seems to be unaffected by the amount of Ca in the diet; the slight tendency to falling off on the lowest Ca intakes is in all probability to be accounted for in the same way as the falling off in the percentage of Ca in the bones produced on the lowest diets. Formulae (2) and (4), for calculating breaking stress in twisting, are probably less satisfactory than the others because of the impossibility of measuring the bone at the actual site of the fracture owing to the spiral character of the break; there is a greater scatter of the results than in the bending tests but there is no indication that the quality is affected by the amount of Ca in the food.

When these experiments were planned it was hoped that it would be possible eventually to apply the results to human material; but since the bone quality is unaffected by the level of Ca intake it is obvious that examination of a sample of human bone would give no information of the previous level of dietary Ca. Whether rachitic changes could be measured by the methods used here has still to be investigated.

The average breaking stresses from our tests in bending and twisting are approximately 35,000 and 9500 lb./in.² respectively. Similar tests on specimens of hard wood, cast iron, and mild steel would give values of 10,000, 40,000 and 70,000 for bending and 1000, 24,000 and 40,000 for twisting. Hence bone material is stronger than timber, nearly as strong as cast iron and half as strong as steel. The ratio of breaking stress in twisting to that in bending is of some interest. For isotropic material, such as cast iron and steel, the ratio is slightly more than a half—but for laminated material like wood the ratio is considerably less, about one-tenth; this is due to the weakness of the cement substance between adjacent lignified fibres. Evidently bone material, with a ratio of 0.27, has some such weakness at the interconnexions between the longitudinal fibres.

We have been able to trace only one investigator who has estimated the breaking stress of bone material, Rauber [1876]. His values are on the average lower than ours, 12 kg./mm.² = 17,000 lb./in.², and are presumably for human material. (It is not to be inferred from this that rat and human bone material are different; the details of the method used by Rauber are not available to us.)

With the actual dimensions and strength of bone material before us it is now possible to consider the ability of a bone to support the body weight when acting as a simple prop. If we take the case of an animal of average weight, say 0.51 lb., on a good Ca diet, which falls to the

these very thin bones. It was extremely difficult to remove this fibrous matter without damage or fracture, and thus these very fragile bones may have been less thoroughly cleaned than the sturdier ones. We are inclined to believe that the actual composition of the bony material was constant throughout the experiment.

Skiagrams

The variations in the thickness of the wall of the femoral shafts were clearly demonstrated in the skiagrams. The bones produced on the lowest Ca intake appeared very much less dense and the trabeculae in the epiphyses were much finer than in the bones produced on the higher diets; one would be inclined on the X-ray appearances to describe the former as atrophied or rarefied. The differences in appearance are due solely to the difference in thickness of the wall and not to any qualitative alteration; the terms atrophy and rarefaction should, therefore, be used very guardedly in describing X-ray appearances in bone until it has been definitely proved by some independent method that there is actually some change in the quality of the material.

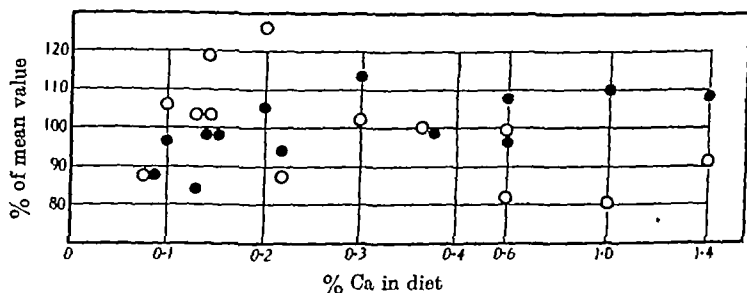


Fig. 9. Breaking stress as percentage of the mean value plotted against percentage of Ca in the diet. Breaking stress in bending, ●; 100 represents 35,000 lb./in.² Breaking stress in twisting, ○; 100 represents 9500 lb./in.²

Strength measurements

The values are given in Table 1, and in Figs. 7 and 9. The strength of the bones as indicated by the bending moment M , and the twisting moment T , increases with increasing Ca intake up to about 0.36 % Ca in the diet; on higher intakes the bones do not become stronger. The meaning of the various formulae used to measure the quality of the bone material has already been explained. The data for evaluation of M/abt are probably the most reliable since the bone broke at the point at which a and b were measured and because t showed less scatter than t' ;

the other three experiments carried out on a different strain of rats. It is obvious, however, that in a more acute Ca deficiency, e.g. produced by a Ca free diet, the initial skeletal Ca, even when redistributed according to the requirements of growth, would ultimately fail to preserve the external dimensions laid down by heredity and failure of growth would result. If the curves of Fig. 7 are extrapolated to zero percentage of Ca in the diet, the curve marked *A* (total Ca retention) would reach zero retention as would be expected; the curve marked *B* would reach 25 % of the maximum, in these circumstances the femoral wall would be reduced to between 4 and 5 thousandths of an inch, part of which would be periosteal tissue. These bones would have only one-quarter of the strength (by extrapolation) of the best bones of our series, but judging from our experience with the bones produced on the lowest Ca intake it would be almost impossible to dissect them out of the animal without fracture.

In our experiments the differences in Ca intake produced variations in the width of the marrow cavity of the bone while the external dimensions remained the same. As the result of experiments on swine Burnett [1908] concluded that no apparent increase in external measurements of the bones resulted when protein or mineral matter was added to the feed but that 'these additional nutriments... have added to the thickness of the bone walls by accretion on the inner surface of these walls, thereby reducing the marrow in the bone'. Bearing in mind the views on bone formation of Brash [1934] this finding may be expressed in a dynamic form as follows. There are two sources of Ca for the formation of bone, (1) the Ca of the food and (2) Ca reabsorbed from the walls of the medullary cavity. The new bone is deposited during growth on the periphery of the shaft of the bone while the older bone is removed from the interior of the shaft; the salts removed in this way presumably circulate and are deposited on the periphery. Ca storage is not comparable to fat storage. Fat stored is material in excess of the body's immediate metabolic needs and the limit of storage is not definitely set. But increase of Ca intake seems to spare Ca reabsorption (or endogenous Ca metabolism) and does not affect the rate of new bone deposition on the periphery because the external dimensions are unaffected by increased Ca intake and because the material of the bones (on whatever diet) is of the same chemical and physical structure. There is a limit set to this sparing action of high Ca intake, otherwise the marrow cavity would disappear entirely on very high intakes. We have no evidence as to how this control is effected, but it can be grossly upset by large doses

ground landing on its hind legs (perhaps a rather remote possibility for a four-footed animal) the impact might increase the weight effect by five times, i.e. to 2.55 lb.; each femur would receive an axial load of 1.3 lb. Taking average dimensions of the shaft section of the bone as 0.120, 0.100 and 0.018 in. for a , b and t , respectively, and the breaking stress of material as 35,000 lb./in.², we find that the strength of the bone as a simple prop is 180 lb. The bone is thus 140 times stronger than is necessary for this action. Our conclusion is that bones are 'made' not as simple props but rather to withstand the severer actions of bending and twisting.

It is perhaps interesting to speculate on engineering principles whether with the material available the bones could have been better designed to resist bending and twisting. A solid rod constructed out of the same amount of material would certainly be much weaker; and a bone of greater diameter with a correspondingly thinner wall would fracture readily on impact and would necessitate more cumbersome joints and more bulky limbs. The tubular bony shaft of the shape and dimensions actually found in the animal seems to be the best for withstanding the forces likely to be applied. Indeed it would be extraordinary if it were otherwise.

It is hardly necessary to apologize for the use of inches and pounds in an engineering problem in place of the more 'scientific' metric units. 1000 lb./in.² equals 0.703 kg./mm.²; the other conversion factors are well known.

CONCLUSIONS

Variation of the Ca intake between the limits used in these experiments did not affect the appetite nor the general condition of the rats, consequently the animals all grew to practically the same length and weight (although there was a tendency noted in some experiments for the gain in weight per gram of food eaten to be greater on the higher diets). The loss of appetite and interference with growth which results from the feeding of diets deficient in certain amino acids and vitamins is not evident in our experiments on Ca deficiency; but the more acute experiments of Kleiber, Boelter & Greenberg [1940] showed that failure of growth and appetite does occur when rats are fed on a diet containing only 10 mg. Ca/100 g. The level of Ca intake in our experiments did not determine the size of the skeleton, as judged from the length of the whole animal or the external dimensions of the femora; it would appear that these are genetically determined—this would account for the discrepancy between the measurements obtained in Exp. I and those of

content of the femora, in the bending and twisting strengths of the femora, and in the thickness of the femoral shell; maximal values of these were reached on a diet containing 0.36 % Ca and further increase of Ca in the diet produced no further change in these values; (3) no change in body length, the external dimensions of the femora, the percentage of Ca in the femora, the quality of the bony material as revealed by the breaking stress in bending and twisting—the items in this group seem to be determined by heredity.

A tentative theory of endogenous and exogenous Ca metabolism as affecting bone formation is discussed. The terms rarefaction and atrophy should be used to describe X-ray appearances of bone only when there is independent evidence of change in the quality of the bone.

There is no harm done to the rat by a diet containing more than 0.36 % Ca but apparently no advantage; lower values of intake may be quite adequate.

The shape and the size of the bones, as found in the animal, are such that the best use is made of the material available to resist bending and twisting. Bone is elastic up to the moment of breaking. It is nearly as strong as cast iron.

We wish to thank Prof. Cathcart and Prof. Wishart for their interest in our work and Prof. Brash for useful information. The expenses were defrayed by the D.C. Andrew Fund of the University of Glasgow.

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of oestrogens [Gardner & Pfeiffer, 1938], suggesting that it is perhaps the pituitary gland which plays a part in preventing the body using all the Ca which is presented to it. This view of the effect of high Ca intake makes it understandable why Ca is stored as living bone and not in some less elaborate form.

This interpretation of our findings is put forward with a certain diffidence; the proof of this dynamic description is beyond the present experimental technique. Further, it is possible that an animal on a low Ca diet followed by a high Ca diet might lay on bone peripherally as well as centrally provided that it is still growing. This question and others might perhaps be studied by the use of isotopes of Ca and P.

It is natural to enquire if we can state from these experiments what constitutes an adequate ration of Ca for the growing rat. It would appear from Fig. 7 that above an intake of 0.36 g. Ca/100 g. dry matter there is no increase in the size, Ca content or strength of the bones. There is no doubt that, as far as Ca is concerned, an intake above this is a *luxus* intake; but it may be assumed that an animal can carry out its ordinary activities on lower intakes. On the other hand, a greatly increased Ca intake seems to do no harm because of the limit set to bone formation. If further increase in thickness had occurred it would have provided very little increase in strength at the expense of increase of skeletal weight—although this is not so important to the rat as to man where the skeleton forms a larger fraction of the body weight. When the marrow cavity is greatly encroached on, as in the experiments reported by Gardner & Pfeiffer [1938], the haematopoietic function is interfered with.

SUMMARY

Seventy-seven male albino rats were subjected to diets varying only in respect of their Ca content (and also in P, though to a somewhat different extent) from 0.075 to 1.390 %, this variation being brought about by varying the proportions of CaHPO_4 and BaSO_4 in the salt mixture.

The reasons for the choice of bending and twisting tests for measuring the strength of bone are given, and the apparatus is described in detail. From the results of these tests the breaking stress, a measure of the quality of the material, can be calculated.

With increase in the percentage of Ca in the diet there was (1) very little change in the total intake of food, with consequently very little variation in gain in body weight in spite of the wide range of Ca intake; (2) a progressive rise in the total Ca retention, in the weight and Ca

transitional stage of a spike-like potential at the end-plate zone, but not at the region of the other electrode which lies across the innervated muscle



Fig. 1.

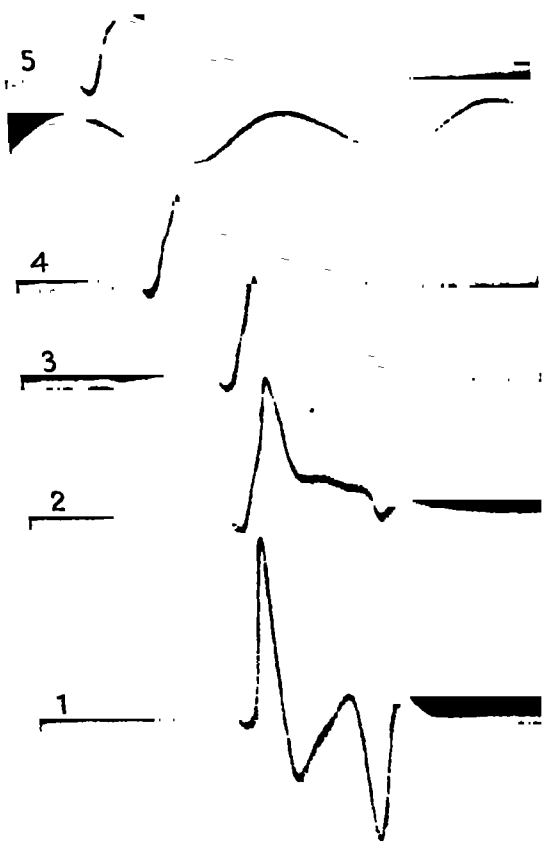


Fig. 2.

Fig. 1. Action potentials set up by a single nerve volley and recorded from innervated soleus strip of cat, one electrode on end-plate zone, other distally on strip. Observation 1, pure e.p.p.; 2 and 3 show propagated spike—probably impulse in a single muscle fibre. Time: 1 d.v. = 10 msec.

Fig. 2. As in Fig. 1 in another experiment. Observation 1, before curarization; 2, 3, 4, 5 show progressive stages in curarization. Observation 5 shows pure e.p.p. but in observations 3, 4 in addition there is a spike-like potential at the end-plate zone which is not recorded at the distal electrode. Time: 1 d.v. = 10 msec.

strip about 18 mm. distal to the end-plate zone (observations 3 and 4). With the weaker curarization of observation 2 the large additional spike at the end-plate zone is propagated as an impulse along some of

ABORTIVE IMPULSES AT THE NEUROMUSCULAR JUNCTION

BY J. C. ECCLES AND W. J. O'CONNOR¹*From the Kanematsu Memorial Institute of Pathology,
Sydney Hospital, Sydney**(Received 18 June 1941)*

In deeply curarized muscle a nerve impulse sets up only an end-plate potential (e.p.p.), which spreads electrotonically from the end-plate zone [Eccles, Katz & Kuffler, 1941]. Between this state of complete paralysis and the initiation of propagated muscle impulses in the partially curarized muscle, a transitional stage has been described [Eccles & O'Connor, 1938] in which a nerve impulse gives rise to a muscle impulse which quickly dies out. A condition in normal muscle possibly parallel to this has also been described when a second nerve volley is just too early to set up a propagated muscle impulse—there is then a spike-like potential which fails to propagate along the muscle fibre [Eccles & O'Connor, 1939]. Further observations relating to these two classes of 'abortive impulse' are described and discussed in this paper.

The observations were made on the innervated muscle strip of the cat's soleus obtained by the section of all branches of the nerve to the soleus except a small twig which ends in a superficial and localized group of end-plates; thus electrical records may be made from electrodes either at the end-plate region or on the strip away from the end-plates. This preparation and the methods of experiment have already been described in detail [Eccles & O'Connor, 1939; Eccles *et al.* 1941].

A. ABORTIVE IMPULSES IN CURARIZED MUSCLE

In some experiments (7 out of 20) during progressive recovery from complete curare paralysis, the first muscle impulse to be set up is propagated the full length of the muscle fibre (observations 2 and 3, Fig. 1). Fig. 2 is typical of the remaining thirteen experiments in showing a

¹ Junior Fellow of National Health and Medical Research Council of Australia.

electrode, as much as 5 % of the uncurarized spike height being thus attributable to abortive impulses. With lead 2 (1.2 mm. distally) about 2 % of the uncurarized spike height is also attributable to abortive impulses. Now with this same disposition of the electrodes the pure e.p.p. with lead 1 was about twice the size of that recorded with lead 2—much the same relationship as is observed for the abortive impulses.

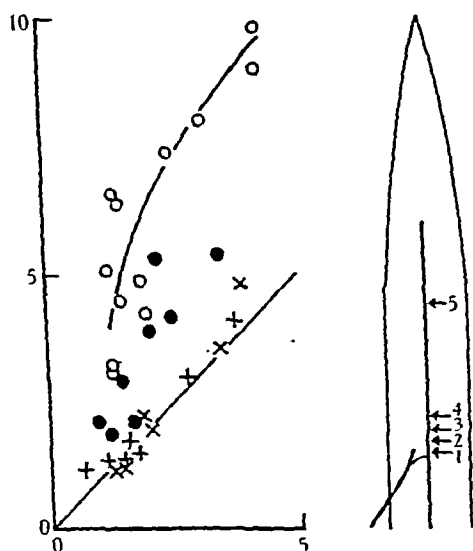


Fig. 3. Diagram shows disposition of electrodes on muscle strip (shown by line on muscle). Electrode 1 is on end-plate zone, and 2, 3, 4 and 5 are respectively 1.2, 2.2, 3.5 and 14.5 mm. distal from it.

Graph. Progressive recovery from curarization: ordinates, size of initial spike recorded with electrodes 1, 2, 3 or 4; abscissae, size of corresponding diphasic spike recorded with electrode 5. Open circles show points for 1-5 leads; closed circles for 2-5; upright crosses, 3-5; oblique crosses, 4-5. All sizes plotted as percentages of corresponding spike size before curarization.

Thus the true impulse propagation of these abortive impulses must be so short that it cannot be distinguished from the propagation by electrotonic spread [cf. Hodgkin, 1937], and it probably is less than 1 mm.

This conclusion also obtains for the seven other experiments where this investigation has been carried out with a series of electrodes close to the end-plate region. The abortive impulse potential was observed in all, and its largest value was about 20 % of the uncurarized peak potential. Further, in every experiment in which there has been a sufficient investigation of the partly curarized muscle, the abortive impulse

the muscle fibres, for it is recorded as a much smaller inverted spike by the distal electrode. The possibility must be considered that with observations 3 and 4 of Fig. 2 the spike was due to impulses propagated along muscle fibres so far away from the distal electrode that no spike response was picked up by it. But against this possibility observation 1 shows that the size (as measured by area) of the spike recorded at the distal electrode in the uncurarized muscle was almost as large as that from the end-plate electrode. These conditions have also obtained in our other experiments in which no diphasic component accompanied the first spike to appear on recovery from curarization. Thus this initial spike cannot be due to muscle impulses which propagate along the full length of the muscle fibres. However, its time course is so similar to that of a normally propagated muscle impulse that it would appear to be due to muscle impulses whose transmission has been blocked at some point along the muscle fibres. Such impulses may be called 'abortive impulses'.

The distance traversed by these abortive impulses has been investigated by following the whole course of recovery from curarization by records from a series of electrodes, one on the end-plate zone and four others on the muscle strip as shown in the inset diagram in Fig. 3. The time course and size of the potential recorded by the most distal electrode in the uncurarized muscle ensure that it is placed right across the innervated muscle strip and well proximal to the tendinous endings of the muscle fibres. This electrode has served as a grid lead throughout, the earthed lead of the amplifier being connected to each of the other electrodes in turn. In this way diphasic records are obtained, the second phase (earth positive to grid) indicating the size of the propagated spike, while the first phase (the negative spike) gives the corresponding spike size at the regions adjacent to the end-plate zone. In Fig. 3 the sizes of the positive spikes are plotted as abscissae against as ordinates the sizes of the corresponding negative spikes for each position of the earthed lead, all spike sizes being calculated as percentages of the corresponding spikes in the uncurarized preparation. With leads 3 and 4 (respectively 2.2 and 3.5 mm. from the end-plate zone) the points lie approximately along the 45° line; hence their recovery resembles that of the distal electrode 14.5 mm. from the end-plate region. Thus any impulse which reaches lead 3, i.e. which propagates about 2 mm., continues to travel along the full length of the muscle fibre. On the other hand, in this early stage of recovery from curarization the curve for lead 1 shows that there is at the end-plate zone a much larger spike than is recorded by the distal

and then flaring up to a fully propagated impulse, as has been observed by Hodgkin [1938, p. 112] for long latency responses in single nerve fibres. This latter explanation would conform with the view that, in the

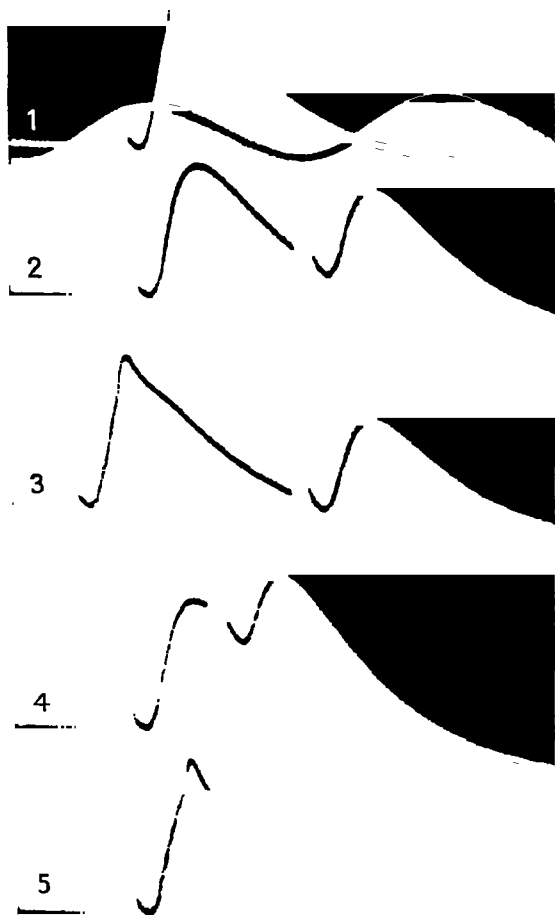


Fig. 4. Action potentials as in Fig. 1, but set up by two nerve volleys in observations 2, 3 and 4. See text.

spread of an impulse from the end-plate zone, a critical stage is reached where it may die out. When just surviving this critical stage, a second spike would be produced by the sudden flaring up of the impulse to the fully propagated size. Double spikes suggestive of such an effect have

potential has been recognized at the end-plate region; and this even in the seven experiments in which the first impulse to appear was propagated the whole length of the muscle (cf. Fig. 1). The abortive impulses are then observed in units which recover from the curarization at a slightly later stage. Thus abortive impulses are set up during the early stages of recovery of some muscle fibres, while other muscle fibres respond by a propagated spike or not at all; and one or the other type of muscle fibre may recover first, the former in thirteen of our experiments, the latter in seven. During the course of some experiments there was a progressive diminution in the abortive impulse potential, which eventually almost completely disappeared, i.e. all muscle fibres then recovered to give propagated impulses without the intermediate stage of abortive impulse.

In all experiments from which a sufficient number of observations is available, there is the definite indication that, if the abortive impulse appears in a muscle fibre at all, it is quite large. In fact, there is a suggestion of an all-or-nothing character in Fig. 4, where the abortive impulse occurs in the response to the first volley in observations 1, 3 and 5, but not in 2 and 4. Thus the abortive impulse would seem to die out only after it had attained a considerable size, i.e. the critical stage for further growth occurs relatively late, and not from the beginning, as with the local response observed in nerve by Hodgkin [1938]. However, in addition, Hodgkin (pp. 110-13) has described a response of some single nerve fibres which is probably analogous to the abortive spike; the local response may spread as much as 1.5 mm. along a nerve fibre and yet eventually die out without flaring up to a fully propagated impulse.

Some light is thrown on the critical size of an abortive impulse by detailed consideration of its time course. In the experiment of Fig. 4 the time course of the abortive spike in observations 1, 3, 5 may be determined by subtracting from them the pure e.p.p. of observations 2, 4. The spike, so determined, is shown in Fig. 5*a*, and exhibits a fairly constant size and time course, though the latent period may vary considerably. With slightly more recovery from the curarization (Fig. 5*b*), (i) the abortive spike is larger, and (ii) a second spike may appear superimposed on the declining phase of the first, or even (iii) on its summit. There is a diphasic dip at a constant interval (5 msec.) from this second spike, indicating that it is propagated along the full length of the muscle. It is possible that the second spike is due to an impulse in another muscle fibre, full propagation occurring with no intermediate stage of abortive impulse. Alternatively, it is due to the initial impulse partly dying away

There is, in addition, a possible transition between types *b* and *a*, the initial impulse at first resembling an abortive impulse and tending to die out, but then flaring up to the fully propagated type.

There is no fundamental subdivision of muscle fibres into those giving types *a* and *b* responses. For example, a curarized muscle has initially been observed to give an abortive spike response as high as 20 % of the peak potential, but, during recovery from a second dose of curare, the abortive spike response was very small, i.e. muscle fibres which originally belonged to type *b* later passed over into type *a*. Therefore it seems likely that in all muscle fibres there is a stage of weakened propagation in the spread of impulses from the end-plate region, but this stage is only demonstrable when this weakening of propagation is adequate either to block or to delay the further spread of the impulses.

B. ABORTIVE IMPULSES IN REFRACTORY MUSCLE

It has been shown [Eccles & Kuffler, 1941*a*] that the pure end-plate potentials set up by an early second nerve volley all have the same time course and so match throughout their whole course provided that allowance is made for differences in latency (cf. observations at volley intervals of 1.1, 1.3, and 1.5 msec. in Fig. 6). At longer volley intervals the muscle spike potential interferes with this over-all matching (cf. the observation at 2.1 msec. interval in Fig. 6). In addition, in some experiments a small spike-like potential interferes with the matching at volley intervals just shorter than that at which the second volley sets up propagated muscle impulses. For example, in the observation at 1.9 msec. interval (Fig. 6), there was no *propagated* muscle spike detectable in the electrical record; and yet at the end-plate zone a negative spike of about 4 % of the peak potential is added *on top of the broken line*, which is the matching curve for the pure end-plate potential at a height indicated by the initial rate of rise of this 1.9 msec. response. There was probably also a small spike-like addition for the response at 1.7 msec. (not shown). This spike-like potential in the absence of propagated muscle impulses has already been described [Eccles & O'Connor, 1939, cf. Fig. 15, observation at 1.83 msec. interval] and attributed to abortive muscle impulses resembling those set up in curarized muscle. That suggestion is supported by the 'matching' experiments, where 'abortive impulses' are usually recorded with volley intervals just shorter than those for propagated spikes. However, in a few experiments abortive

been observed in five of our experiments. However, distinction between the above two explanations would only be possible with single muscle-fibre preparations.

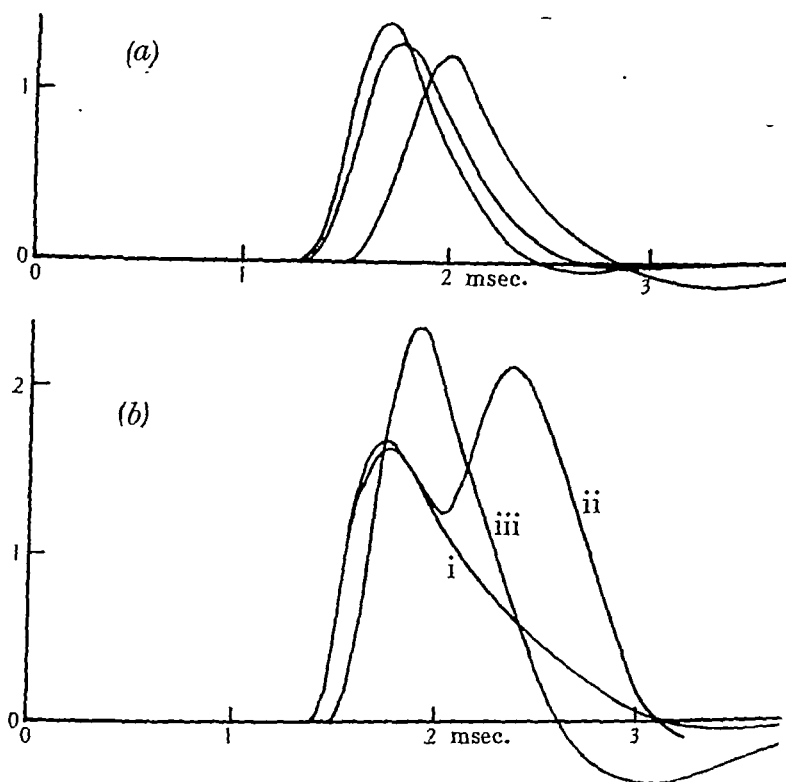


Fig. 5a. Three abortive spikes of Fig. 4 as determined by subtraction of e.p.p. Ordinates expressed as percentage of uncured peak potential for innervated strip: abscissae, time after nerve stimulus. Fig. 5b, as in Fig. 5a, but with further recovery from curarization. *i*, is an abortive spike; *ii*, initially resembles an abortive spike but later there is a propagated spike; *iii*, is a propagated spike.

Conclusions

During recovery from curarization, the first muscle impulses to be set up by a nerve volley may belong to either of two types:

(a) Fully propagated impulses which spread with a normal velocity from the end-plate zone along the muscle fibres.

(b) Abortive impulses which die out after a very short spread, probably less than 1 mm. They form a transitional phase between complete neuro-muscular block and type *a*.

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THE GLYCOGEN CONTENT OF THE FROG'S HEART

BY F. DAVIES AND E. T. B. FRANCIS

*Departments of Anatomy and Zoology, University of Sheffield.
With a Chemical Section by L. B. WINTER, Department of Physiology*

(Received 28 June 1941)

FOR the mammalian heart (dog), Lewis [1925] has expressed as 'The Law of Cardiac Muscle' a correlation between the size (breadth), glycogen content and physiological properties of cardiac muscle fibres, namely, that the fibre size, glycogen content, and rate of conduction of the impulse for contraction increase in the order, nodal, ventricular, atrial, Purkinje tissue; while the length of systole and property of rhythmic contraction (when nourished under natural conditions) diminish in the same order.

There has, however, been some controversy as to whether the rapidly conducting Purkinje fibres in the mammalian bundle of His do actually contain more glycogen than the ordinary cardiac muscle. By histological study, after staining with either Best's alkaline carmine or iodine, Mönckeberg [1908], Nagayo [1908], Lewis & Rothschild [1915] and Ungar [1924] claimed that the Purkinje system is richer in glycogen. On the other hand, Buadze & Wertheimer [1928], by quantitative chemical estimations, found less glycogen in the Purkinje fibres than in the general myocardium (dog, goat, sheep) and thus concluded that the histological methods are unreliable. The later careful quantitative estimations of glycogen by Yamazaki [1929] on the horse and ox, by Yater, Osterberg & Hefke [1930] on the horse, and by Noll & Becker [1936] on the horse and calf, however, all agree that the bundle of His is richer in glycogen than the myocardium. Indeed, Noll & Becker employed the carmine and iodine histological tests alongside their quantitative chemical estimations, and found that the results agreed. These later studies thus substantiate the reliability of the histological methods.

with longer volley intervals the spike origin encroaches progressively earlier on the e.p.p. [cf. Eccles & Kuffler, 1941*a*, Fig. 13], and thus may be partly responsible for effect (i).

SUMMARY

Between complete neuro-muscular block and normal neuro-muscular transmission there is often a transitional stage in which a nerve volley sets up muscle impulses which die out after a very short propagation—probably less than 1 mm. These 'abortive impulses' are observed when neuro-muscular transmission is impaired either by curarization or refractoriness of the muscle.

In the discussion reinterpretations of some of our earlier findings are suggested.

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Histological

The hearts of 3 frogs (*Rana temporaria* L.) were rapidly removed from pithed animals and immediately placed in abs. alcohol. In this connexion it may be noted that while Evans [1934] showed that in the mammal (rat) asphyxia (anoxaemia) causes rapid reduction in the glycogen content of the heart, Clark *et al.* [1938] found that even prolonged manipulation (10–15 min.) under conditions of anoxaemia causes no appreciable diminution in the glycogen content of the frog's heart. The 3 hearts were embedded in paraffin and cut transversely in serial sections at 5μ thickness, and stained with alkaline carmine [Best, 1907], controlled by the saliva test and by sections of liver stained at the same time.

On visual inspection with the microscope, it was at once apparent that the glycogen content of the muscle fibres differ in the several chambers of the heart. The sinus muscle is not very rich in glycogen; the atrial muscle contains slightly more; the ventricular muscle is considerably richer than the atrial, while the bulbar muscle contains still more. Further, the glycogen content of the musculature at the sinu-atrial and atrio-ventricular junctions shows no appreciable difference from that of the neighbouring chambers. It thus appears that the glycogen content of the muscle fibres shows a progressive increase from the sinus to the bulbus.

Chemical estimation of glycogen

Alongside our direct histological observations, chemical investigation has been carried out in collaboration with our colleague, Dr L. B. Winter, of the Physiology Department, University of Sheffield. Two series of estimations were made, 6 animals being used for each.

First series. Each heart was rapidly removed from a pithed animal and placed immediately in an ice-cooled glass tube. The several chambers were successively separated from each other by section and, after removing adherent blood with filter paper, were weighed on a micro-torsion balance; the operation being so arranged that individual parts of the hearts were out of the ice-cooled tube for only a very brief period. In the case of the sinus and bulbus, owing to their small mass, the entire chambers were used, but in the case of the ventricles and atria, portions only of the walls were removed and weighed, the parts removed differing in each heart so that all parts of these chambers were represented in the final mass of tissue on which the estimation was made. After weighing,

So far as lower vertebrates are concerned, Clark *et al.* [1938] maintain that the number of chemical estimations that can be made on so small a quantity of tissue as that provided by frogs' hearts is limited, and these authors preferred to estimate the total reducing substances rather than the glycogen alone. Such estimations include both glycogen and lower carbohydrates, as well as some reducing substances which are not carbohydrates; for the sake of convenience, although as they point out it is not strictly accurate, they employ the term 'total carbohydrates' to cover all these substances. Since by an analysis of nine individual tortoise hearts they found [1933] only a small difference between the total carbohydrate content of the atria and ventricle (average—atria 1.5 %, ventricle 1.67 %) they concluded that 'one portion of the frog or tortoise heart, therefore, will serve as a fairly reliable control as regards the carbohydrate content of the remainder'. Various observers, using chemical methods, have noted considerable individual variation in the carbohydrate content of frogs' hearts. A seasonal variation also has been noted by Wertheimer [1933] and by Clark *et al.* who find more carbohydrate in the hearts of winter than summer frogs.

The present authors [Davies & Francis, 1941] have shown that there are no differences—apart from size—in the histological structure of the muscle fibres of the various chambers of the heart of either the salamander or frog (sinus, atria, ventricle, bulbus), as revealed by staining with haemalum and eosin, Van Gieson's acid fuchsin and iron haematoxylin, or with Bodian's activated protargol. They also demonstrated a similar absence of histological specialization of the musculature joining the chambers of these hearts, and concluded, contrary to the view commonly held, that the Purkinje system of the mammalian (and avian) heart is a neomorphic structure, and is not derived from more extensive junctional tissues of similar structure in lower vertebrate hearts.

The aim of the present investigation is to determine whether in one and the same frog's heart there are any differences in the glycogen content of the musculature of the several chambers or of that at the junctional sites.

OBSERVATIONS

For both the histological and chemical investigations the hearts were removed early in February from animals which had been kept in captivity for some months.

It will be seen that whereas in the case of the sinus, atria and ventricle these results are slightly higher than those of the first series, the percentage of glycogen in the bulbus in the second estimation is more than three times that found in the first experiment.

Graphical method of estimation

Owing to the difficulty of obtaining a valid estimate of the glycogen in the *musculature* alone in the bulbus by chemical means, we have estimated the *relative* amounts of glycogen in the musculature of the several cardiac chambers by a graphical method. The histological sections, stained with Best's carmine, were projected by means of an arc lamp and microscope on to transparent graph paper, ruled in millimetre squares, at a linear magnification of $\times 1480$. The outlines of small areas of cardiac muscle (in longitudinal section) from each chamber were traced and within these frames the outlines of the red-stained globules and small masses of glycogen were mapped out. By counting the number of squares covered by both the muscle and the glycogen a ratio was obtained which serves to indicate the relative amounts of glycogen present in the musculature of the various parts of the heart. (The reliability of this method is discussed below.)

By this means the following results were obtained, expressed as fractions in which the numerators represent the number of millimetre squares covered by glycogen and the denominators the total areas of cardiac muscle fibres outlined: sinus, $18,182/156,475 = 11.62\%$; atria, $28,428/200,406 = 14.18\%$; ventricle, $50,211/175,307 = 28.6\%$; bulbus, $67,549/218,008 = 31.03\%$. The musculature at the sinu-atrial and atrio-ventricular junctions was analysed in a similar manner and the following results obtained: s-a ring, $17,349/123,980 = 13.99\%$; a-v ring, $41,120/145,935 = 28.2\%$; a-v funnel, $31,120/127,895 = 24.3\%$. Each of the above results represents the total of many tracings made from sections of different regions of the several cardiac chambers, so that the total represents a fair average of the glycogen content of the musculature of the chamber concerned. It was noted in making individual tracings of small areas of each chamber that, although there was a fair consistency in the amount of glycogen in each area, there was, however, considerable indiscriminate variation in the glycogen content of neighbouring fibres.

the fragments were placed in ice-cooled, small centrifuge tubes and corked, as follows: tube A, 38 mg. bulbus; tube B, 38 mg. sinus; tube C, 39 mg. ventricle; tube D, 35 mg. atria.

Chemical estimation by L. B. WINTER

The tissue samples were handed to me in small centrifuge tubes, corked and cooled in ice: the tubes were numbered and the weight of tissue in each was stated, but I was unaware which tissue was contained in each tube until the estimations had been completed and the percentage of glycogen (as glucose) in each tissue calculated. For the estimation of glycogen the directions given by Osterberg [1929] were followed, with certain modifications. The weight of tissue was greater, and to each tube was added 0.2 c.c. of 60 % potash. After 3 hr. reflux under an air condenser at 100° C., the tubes were allowed to cool in the bath and to each were added 0.2 c.c. water and 1.2 c.c. strong alcohol: the contents were well mixed. Five days in a refrigerator were allowed for complete precipitation of the glycogen; by this means the precipitate was induced to adhere firmly to the bottom of the tubes, and the use of the centrifuge was always found to be unnecessary during the successive washings. Each day the supernatant fluid was carefully poured off, the tubes being inverted for a few minutes to ensure complete draining, and 2.0 c.c. 70 % alcohol were run in. After one washing each day for 5 days the alcohol no longer coloured phenolphthalein and the tubes were dried in vacuo over sulphuric acid. For the conversion of glycogen to glucose, 2.0 c.c. 2.2 % HCl were added to each tube, and reflux at 100° C. was carried out for 3 hr. After cooling, the exact amount of alkali was added to neutralize the acid; the contents were stirred, transferred to a 10 c.c. volumetric flask and the tube was washed out with small amounts of water. 4.0 c.c. portions were taken for duplicate glucose estimations by the method of Hagedorn and Jensen. Results: sinus, 0.15 %; atria, 0.62 %; ventricle, 1.52 %; bulbus, 0.22 %.

It will be observed that whereas these results agree with our visual observations as far as the increasing order of glycogen content of the sinus, atria and ventricle is concerned, there is a marked discrepancy in the case of the bulbus. This would appear to be due to the relatively large amount of connective tissue present in the bulbus, including the fibrous septum bulbi and valves, which would have the effect of diminishing the proportion of glycogen-containing muscle in the weighed amount of bulbar tissue, thus giving too low a figure for the chemical estimation. This idea is supported by the results obtained in the second series of estimations.

Second series. The procedures detailed above were repeated with another 6 frogs except that the septa were removed from two bulbi. The remaining four bulbar septa were not removed since, owing to the way these bulbi contracted on excision, the operation was deemed likely to require too much time and to involve too much traumatic injury. The two bulbi from which the septa were removed offered very favourable conditions and the operation required only a few seconds. Results: sinus, 0.26 %; atria, 0.78 %; ventricle, 1.85 %; bulbus, 0.73 %.

glycogen content and the intrinsic rhythmic rate of a cardiac muscle fibre, or is purely coincidental, remains to be determined. A further correlation can, however, be made in that the density of musculature and the work done increase in the same order as the glycogen content of the muscle fibres, suggesting that the increasing glycogen content is related to the greater energy demands for the work of contraction in the successive cardiac chambers.

Now with regard to the specialized conducting system of the mammalian heart, the Purkinje fibres contain more glycogen than the ordinary myocardium. This Purkinje system has been held by various workers to represent remnants of more extensive tissues of similar structure in the hearts of lower vertebrates. The present authors, however, as the result of a detailed histological study of the amphibian (salamander) heart, have elsewhere [1941] put forward the view that the cardiac conducting systems of homoiothermal vertebrates (mammals and birds) constitute neomorphic developments, and are not remnants derived from the lower vertebrate heart. They based their conclusions on the complete lack of any histological specialization in the musculature joining the several cardiac chambers. The absence of any accumulation of glycogen in the junctional muscle of the frog's heart affords additional evidence in favour of this view.

It should be emphasized that the present work is concerned solely with the *relative proportions* of glycogen present in the *musculature* of the various parts of one and the same heart at a given time, and not with the absolute quantities of glycogen in each chamber, or with the variations due to seasonal or other disturbances which are known to occur.

SUMMARY

1. Visual examination of serial sections of frogs' hearts, stained by Best's carmine method, revealed a progressive increase in the glycogen content of the muscle fibres, chamber by chamber, from sinus to bulbus. Graphical analysis confirmed this. Chemical analysis of the separated chambers agreed with the visual and graphical results, except in the case of the bulbus, where the figures were much lower than anticipated; this is explained by the relatively large amount of connective tissue in the bulbus.

2. The glycogen content of the muscle fibres is correlated directly with the work done by the several cardiac chambers, and inversely with their intrinsic rhythmic rates.

DISCUSSION

Of the methods described above, chemical estimation is evidently the only one which provides an absolute figure for the amount of glycogen present in each cardiac chamber. Where, however, there is a considerable amount of connective tissue present (a circumstance only determinable by histological control), the result obtained does not represent the amount of glycogen present in the *musculature* of that chamber. Thus, in the present instance, where it is desired to compare the glycogen content of the *musculature* of the various cardiac chambers, the chemical method is not applicable on account of the varying extent to which connective tissue participates in the structure of the walls of the several chambers of the heart.

The graphical method, on the other hand, does enable a comparison to be made between the glycogen content of the muscle fibres from various parts of the heart. It is obvious that this method depends for its reliability upon the specificity of the carmine as a stain for glycogen. That this may be trusted is indicated by the work of Noll & Becker [1936], while we have ourselves controlled the staining of our material by the saliva test. Further, it must be pointed out that this method deals purely with relative areas, and not volumes, of glycogen and muscle. In any particular individual mass of glycogen outlined, there are parts which do not extend throughout the entire thickness of the 5μ sections, and high magnification reveals that these masses are aggregations of smaller globules. In plotting such masses on squared paper it is necessarily assumed that the areas are homogeneous and extend through the entire thickness of the section. These errors, however, are common to all chambers and therefore do not seriously affect the validity of the results so far as a comparison of the glycogen distribution in the various parts of the heart is concerned, although they do prevent an estimate being made of the absolute amount of glycogen present. On the other hand, the consistency of the results calculated from each of the numerous tracings made from individual cardiac chambers confirm the reliability of this method when used purely for comparative purposes.

With regard to the significance of the results obtained, it is evident that there is, in the frog's heart, a relationship between the glycogen content and the intrinsic rate of rhythmic contraction of the various chambers, in that, while this rate *decreases* in the order sinus, atria, ventricle, bulbus, the glycogen content of the muscle fibres *increases* in the same order. Whether this indicates a causal relationship between the

OBSERVATIONS BEARING ON SYNAPTIC
TRANSMISSION BY ACETYLCHOLINE
IN THE SPINAL CORD

By EDITH BÜLBRING AND J. H. BURN

From the Department of Pharmacology, Oxford

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AMONG the papers published on humoral transmission during the last ten years there have been several in which observations have been made suggesting that acetylcholine acted as a transmitter in the central nervous system. Dikshit [1934] was the first to carry out experiments intended to throw light on this possibility; he showed that small doses of acetylcholine injected into the cerebral ventricle of cats had a similar effect on respiration to that produced by stimulation of the central end of the vagus. He examined the cerebrospinal fluid before and after stimulation of the central end of the vagus to see if acetylcholine was liberated, but was unsuccessful in observing this, except occasionally.

Hitherto no workers have succeeded in obtaining evidence along each of the three main lines generally accepted as indicating humoral transmission by acetylcholine in other structures. When investigating neuromuscular transmission, for example, Dale and his co-workers showed that small doses of acetylcholine given by close arterial injection caused contraction of striated muscle; they studied the action of eserine and related drugs and showed that their effects on muscle stimulated through its nerve were best explained by cholinergic innervation; finally, they demonstrated that when the nerve impulse reached the muscle, acetylcholine was liberated.

In the work described in this paper it has been our endeavour to treat the problem of transmission in the spinal cord along these lines. We have injected acetylcholine into the spinal cord to observe whether it produced a discharge of motor impulses; we have studied the action of eserine and related drugs on the flexor reflex and on the knee-jerk; finally, we have looked for the liberation of acetylcholine in the venous effluent from the cord when stimulation was applied to the central end of the sciatic nerve.

3. The lack of accumulation of glycogen in the junctional muscle supports the authors' view that the specialized cardiac conducting systems of mammals and birds are not remnants of more extensive tissues of similar structure in lower vertebrate hearts.

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tendon of the quadriceps was detached from the tibia together with a piece of bone; this was attached by wire to a tension lever. For the flexor reflex the tendon of tibialis anterior was detached from its insertion and attached to a tension lever, and the posterior tibial nerve was also dissected for a length of about 3 cm.

Small artery clamps were placed on the right external iliac artery and vein near the bifurcation, and cannulae were tied in these vessels pointing towards the bifurcation. Loose ligatures were placed around the aorta and inferior vena cava as high up in the abdomen as possible. Mass ligatures were placed in position to enclose the muscles of the body wall and those surrounding the spinal column. When tied these ligatures were situated about 3 cm. above the left suprarenal gland.

Blood was then withdrawn from a third dog under ether anaesthesia; injections of Ringer's solution were not made into it. When the dog was dead, its lungs were prepared for perfusion, and this was begun with a Dale-Schuster pump [1928]. Another pump was then filled with blood and connected to the cannulae in the external iliac artery and vein of the second dog. When all was ready the ligatures round the aorta and vena cava of this dog were tied, and perfusion of the lower half of this dog was simultaneously begun, the blood being oxygenated by the pump-lung circuit which had already been prepared. The mass ligatures round the body of the dog were tied and the dog was divided above them. The hinder end of the dog was placed in a large glass dish in which leakages of blood were collected. An artificial arterial resistance of the pattern used in the Starling heart-lung preparation was tied into the aorta just above the suprarenal glands. This was introduced merely as a safeguard against excessive rise of pressure from vasoconstriction and the pressure in the resistance was set above the usual perfusion pressure. The lungs in the fore-part of the dog were now prepared for perfusion, and perfusion was begun by a third pump to oxygenate the blood in the leg circuit. The left external iliac artery and vein were next divided between two ligatures at their origin, and perfusion from a fourth pump was begun in the left leg through cannulae tied in these vessels.

In eliciting the knee-jerk the tapper described by Schweitzer & Wright [1937*a*] was used. In stimulating the posterior tibial nerve we used condenser discharges at the rate of 96 per sec. from a neon lamp circuit, and to obtain the flexor reflex these were applied six times a minute, the duration of each application being 0.2 sec. The electrodes on which the posterior tibial nerve was placed were silver plates 3 mm. wide and 1.4 cm. apart; they were shielded in the curved prongs of a vulcanite

THE METHOD

Various problems presented themselves in studying the activity of the spinal cord. Using, as we have, the contraction of muscles as indicator of central activity, it was of first importance to ensure that the action of drugs was confined to the spinal cord and not exerted on the muscles directly. Therefore we devised a system in which there were two circulations of perfusing blood, the one supplying the spinal cord, and the other the muscles.

Another complication to be avoided was the influence exerted on spinal reflexes by the higher centres. We therefore divided the cord in the lower thoracic region and then, to simplify the circulation, removed the upper part of the body altogether. The spinal cord prepared in this way is, of course, abnormal in that it suffers from the effects of acute transection. We have, however, found no difficulty in eliciting the reflexes we wished to study.

DETAILS OF THE PREPARATION

Three dogs were used for each experiment. From the first, blood was drawn from the carotid artery under ether anaesthesia and defibrinated; at intervals during the collection warm Ringer's solution was run into the jugular vein to increase the volume of blood obtained. A second dog was then anaesthetized with ether followed by chloralose. It was eviscerated, the kidneys were removed, and the arteries and veins of the body wall were tied on both sides. The right external iliac artery and vein were divided near Poupart's ligament and dissected up to the bifurcation of the aorta; the aorta was tied below the origin of the external iliac arteries; the last pair of arteries passing back from the aorta to the vertebral column were tied; the internal iliac veins of both sides were tied and divided. The muscles of the anterior abdominal wall were divided above Poupart's ligament on the left side. The left external iliac artery was then dissected and all branches were tied from the origin to a short distance below Poupart's ligament, care being taken to divide branches running up the anterior abdominal wall. The left external iliac vein was similarly prepared. This completed the dissection when the knee-jerk was to be recorded. When the flexor reflex was to be recorded, the femoral artery and vein were dissected free in the thigh and mass ligatures were placed in position round the muscles of the thigh but so as to exclude these vessels and the sciatic nerve. A hole was then drilled through the lower end of the left femur and a steel rod inserted. For the knee-jerk the

Experimental observations

When acetylcholine was injected into the arterial cannula carrying blood to the spinal cord, we observed contractions in the muscles of the separately perfused leg. In the absence of eserine the dose of acetylcholine necessary was large and variable, being from 0.06 to 1.0 mg. This variation we discuss below. If acetylcholine is the humoral transmitter capable of setting up impulses in anterior horn cells it should be possible to obtain these effects with much smaller doses. The distance between the cannula and the cord was, however, large enough to make likely the destruction of most of the acetylcholine before it arrived. We

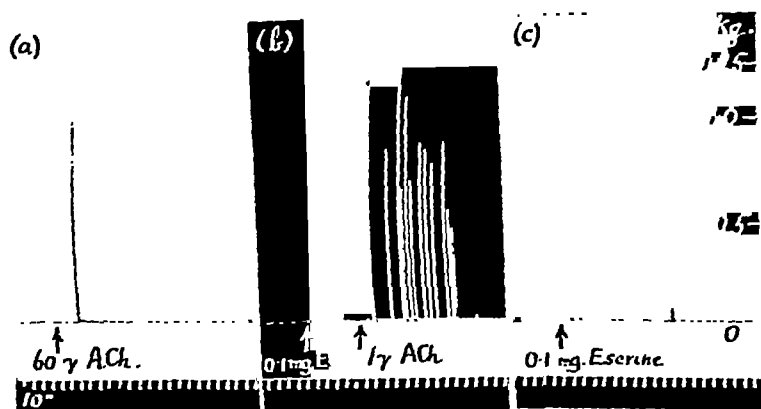


Fig. 1. Dog, double-perfusion system. Record of contractions of *m. tibialis anterior* evoked by injection of acetylcholine into the circulation through the spinal cord. (a) before eserine; (b) series of contractions produced by 1 μ g. acetylcholine after 0.1 mg. eserine; (c) effect of 0.1 mg. eserine.

have not yet succeeded in working out a method of close arterial injection, and have confined ourselves to retarding the destruction of acetylcholine with a preliminary dose of eserine. The method of introducing eserine into the system was important; it was not sufficient merely to add a dose even as great as 1.5 mg. to 600 c.c. of blood (to produce a concentration of 1 in 400,000); moreover, so much eserine had the disadvantage of producing spontaneous discharges on its own account. Under such conditions the smallest effective dose of acetylcholine was 10–20 μ g. Effects such as that shown in Fig. 1, in which 1 μ g. acetylcholine caused a series of contractions in *tibialis anterior*, were obtained in the following manner. In the absence of eserine 60 μ g. acetylcholine caused a single contraction after a latent period of 25 sec. (Fig. 1a); half this dose was

fork. At the beginning of each experiment dextrose was added to the blood in the proportion of 1 g./l., and adrenaline in the proportion of 0.05 mg./l. During the perfusion, adrenaline (1 in 200,000) was dropped into each arterial reservoir in order to control the vascular tone. This was always maintained rather higher in the leg circuit than in the cord circuit, in order that there should be an additional barrier against diffusion from the cord to the periphery. The rate at which the drops of adrenaline entered the reservoirs was adjusted from time to time according to the pressures indicated by the manometers.

SECTION I. THE DISCHARGE OF IMPULSES FROM THE CORD BY INTRA-ARTERIAL INJECTION OF ACETYLCHOLINE

Reference has already been made to Dikshit [1934], who observed that intraventricular injection of acetylcholine caused an effect on respiration similar to that of central vagal stimulation. In 1936 Henderson & Wilson carried out similar experiments in men, finding that large doses of acetylcholine (2.5–7.5 mg.) caused a series of effects resembling those described earlier by Cushing [1932] after the intraventricular injection of pilocarpine; these effects were nausea, vomiting, peristalsis, sweating flushing and sometimes sleep. Bonnet & Bremer [1937*a*] observed the effect of injecting acetylcholine into the carotid artery on the electrocorticogram of cats in which the brain stem was divided just above the pons. Doses of 0.1–0.2 μ g. increased the amplitude and frequency of the waves, while doses greater than 0.5 μ g. depressed them. None of these workers, however, described effects in skeletal muscle due to central action of acetylcholine, and the earliest suggestion of this appears in a paper by Feldberg & Minz [1932] who stated that when large doses of acetylcholine were injected into spinal cats after atropine, muscular twitchings were observed which were mainly central in origin 'since they disappeared for the most part when the cord was destroyed'. Many peripheral effects of injected substances are, however, diminished by destruction of the spinal cord with the consequent slowing of circulation rate.

The effect of acetylcholine on the eserinizied cortex of the rabbit (under procaine anaesthesia) was studied by Miller, Stavsky & Wootton [1940]. A solution (1 %) applied on a piece of blotting paper produced a series of large, rapid spikes in the electrocorticogram accompanied by motor effects in the limbs; similar observations on the lobus anterior of the cerebellar cortex were made. Alterations in limb tonus were again seen, accompanying changes in the electrocorticogram.

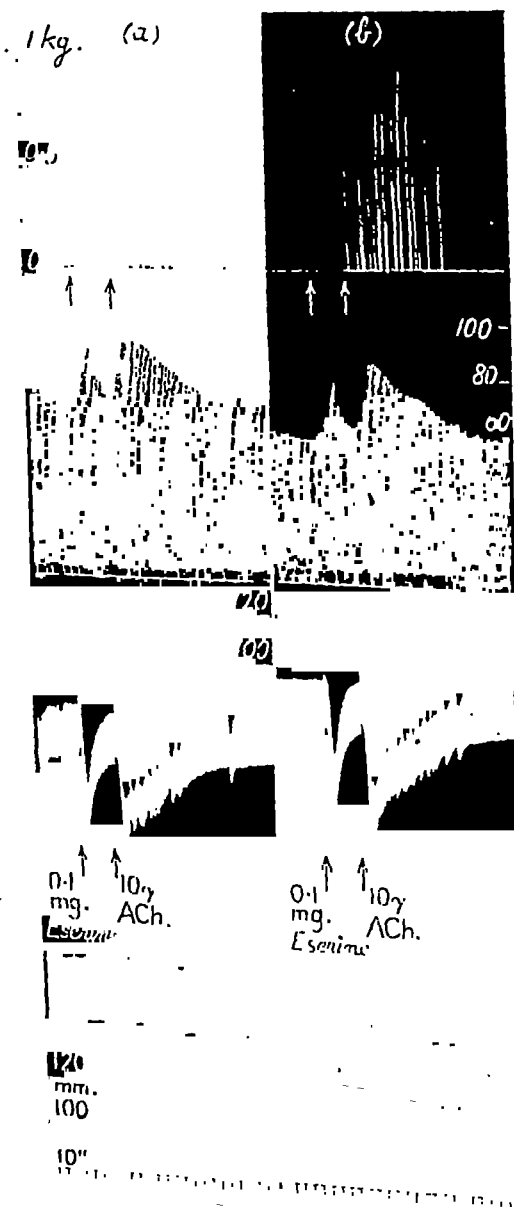


Fig. 2. Double perfusion. Records from above downwards; contractions of m. tibialis anterior; venous outflow (c.c./min.) (Gaddum's recorder) from cord circulation; arterial pressure in cord circulation; arterial pressure in leg circulation. (a) shows that 10 μ g. acetylcholine injected into cord circulation caused no motor discharge. 0.1 mg. adrenaline was then slowly infused after which (b) 10 μ g. acetylcholine set up a series of motor impulses to the leg.

without effect. Eserine sulphate, 0.1 mg., was then injected, after 1 min. by $10\mu\text{g.}$ acetylcholine. This produced one contraction. Minutes after the eserine injection, a second injection of 0.1 mg. was given, followed after 1 min. more by $1\mu\text{g.}$ acetylcholine. This produced after 15 sec. a series of thirteen contractions (Fig. 1b). The effect of the injection of 0.1 mg. eserine alone, without acetylcholine, was not observed and this, as shown in Fig. 1c, produced one very small contraction after a latency of 2 min. Since the effect of eserine given at intervals increases with successive injections rather than decreases, it is certain that the contractions in Fig. 1b were entirely due to acetylcholine.

The effect of adrenaline

In the course of these experiments we were puzzled by the variation in the amounts of acetylcholine necessary to elicit contractions, and we discovered that the effectiveness of the acetylcholine was influenced by the amount of adrenaline present in the blood circulating through the spinal cord. For example, at a later stage of the experiment just described $10\mu\text{g.}$ acetylcholine was ineffective; the arterial tone was low and the venous outflow from the spinal cord was 75 c.c./min. As shown in Fig. 2a. Adrenaline (1 in 200,000) was then added drop by drop to the venous reservoir and the venous outflow reduced to 60 c.c./min. As Fig. 2b shows, $10\mu\text{g.}$ acetylcholine then became effective. At the time when $1\mu\text{g.}$ was effective (Fig. 1b) the tone was higher still and the venous outflow only 50 c.c./min.

We conceive that acetylcholine sets up impulses in anterior horns of the spinal cord. The effect of adrenaline in facilitating such an action could then be explained by supposing that it lowered the threshold concentration of acetylcholine, in the same way as adrenaline lowers the threshold for the transmission of impulses across the neuromuscular junction [Bülbring & Burn, 1940]. There is, however, another possibility; we have also suggested [Bülbring & Burn, 1939] that the transmission along the nerve of electrical stimuli applied to the motor roots depends on the vascular tone, a condition most readily improved by the addition of adrenaline to the blood. Bülbring & Whitteridge [1941] have since found that adrenaline lowers the threshold in peripheral nerve to submaximal stimuli. The explanation of the observations shown in Figs. 2a and 2b may therefore be that in the absence of adrenaline from the blood (Fig. 2a) impulses set up

SECTION II. THE ACTION OF ESERINE AND OTHER DRUGS ON THE FLEXOR REFLEX AND THE KNEE-JERK

The effect of blood flow

Before describing the action of drugs on reflexes, certain general observations concerning the double perfusion system must be made. We found it important in the first place to maintain a tone with adrenaline in the leg circuit, since without it the reflexes were weak. This weakness was due either to inefficient neuromuscular transmission, or to defective transmission along the nerves as already described.

Since the substances investigated included adrenaline and choline derivatives which altered the blood flow through the cord circuit, it was necessary to learn how far alterations in blood flow in this circuit affected the reflex response. Fig. 3 is a record of the knee-jerk elicited at intervals of 10 sec.; it shows that after the injection of 0.3 mg. acetyl- β -methylcholine, when the blood flow increased from 70 to 120 c.c./min., and after the injection of 0.02 mg. adrenaline when the blood flow decreased from 85 to 27 c.c./min., the strength of the reflexes was not affected. These observations do not mean that in other circumstances adrenaline and acetyl- β -methylcholine are inactive.

If the pump stroke in the cord circuit was increased or decreased, the reflexes were unaffected; if it was stopped, the reflexes increased by 50–100% within 10 or 20 sec. and then as rapidly disappeared after a further 30–40 sec.

The action of adrenaline

Earlier observations on the effect of adrenaline on spinal reflexes are few. Schweitzer & Wright [1937b], recording the knee-jerk of the cat under chloralose, stated that this reflex was depressed by adrenaline, though the depression was sometimes preceded by augmentation; Bonvallet & Minz [1938], working on the frog, rat, cat and dog, found that adrenaline depressed reflexes in spinal animals, but in thalamic animals adrenaline increased the excitability of reflexes.

Experimental results with adrenaline

In observations on the knee-jerk the injection of adrenaline into the cord circuit in doses below 0.1 mg. had no effect whatever. Although the blood flow was often greatly reduced the reflexes remained absolutely unchanged. Doses of 0.1 mg. or more regularly caused augmentation of the knee-jerk as shown in Fig. 4, sometimes followed by a slight decrease. It was often surprising that when the blood flow was as much reduced

excitability to electrical stimuli, but also the transmission along nerve of impulses set up within the motor cell.

We have then two possibilities to explain the effect of adrenaline (1) that it produces a lowered threshold to acetylcholine acting on motor cell, (2) that it improves the transmission of impulses from the motor cell along the motor fibre. Whichever explanation is correct, we believe a new and important property of adrenaline is indicated. A further illustration of this property is given below in the effect of adrenaline on the flexor reflex, and also on the effect of prostigmine on that reflex.

The central action of eserine, prostigmine and nicotine

That eserine exerts some stimulant action on the central nervous system has been known for a long time; Langley & Kato [1915] reported the action of physostigmine in causing irregular contraction of the heart and say that these are of central origin and are stopped by atropine. In 1937 Miller examined the action of eserine on the cerebral cortex of the frog and found that the local application of minute amounts of 1% solution to the motor area evoked muscular effects in a few seconds. He concluded that cerebral synapses were facilitated and stimulated by the eserine and thus motor effects were induced. Miller *et al.* [1940] extended his observations by recording the changes in electrical potential. In 1938 Bonnet & Bremer showed that when 5 μ g. nicotine was injected into the aortic arch of a frog a prolonged spontaneous discharge from the spinal centres occurred, as seen in the electromyogram. Chute, Feldberg & Smyth [1940] perfused the isolated cat's brain with 50% defibrinated blood. The addition of eserine to it produced increased reflex excitability and spontaneous movements of the muscles of the head, such as blinking and twitching of the face.

Experimental results

In our experiments in which eserine, prostigmine and nicotine have been injected into the arterial cannula taking blood to the spinal cord, we have observed that all three substances, when given in sufficient dose, caused a discharge of impulses from the spinal cord, which were recorded as contractions of the quadriceps or tibialis anterior. The dose necessary was always larger than the dose which influenced reflexes, and was usually about 1.0 mg. for eserine and 2.0 mg. for prostigmine. We have not observed that nicotine caused spontaneous contractions except when either eserine or prostigmine had been given previously, but when that had been

by the adrenaline as in Fig. 4, the reflexes were not affected by this reduction.

Since these findings did not confirm the results of Schweitzer & Wright [1937b], we repeated their experiments, recording the knee-jerk in cats under chloralose. The only difference between our method and theirs was that we detached the tendon of the quadriceps from the tibia, and

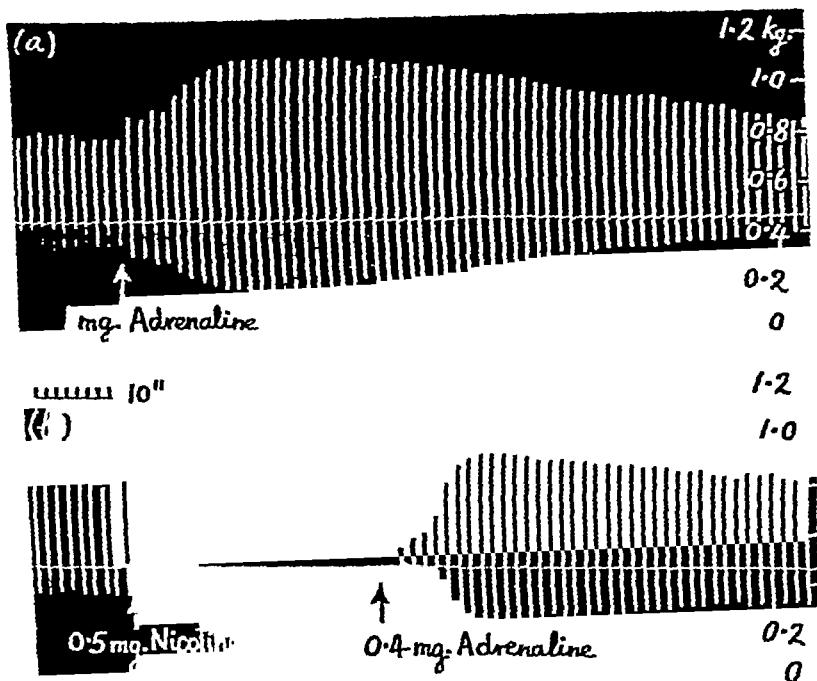


Fig. 5. Cat, chloralose, knee-jerk. (a) shows the augmentation of the knee-jerk after intravenous injection of 1 mg. adrenaline. (b) shows the abolition of reflexes by intravenous injection of 0.5 mg. nicotine, and their recovery after 0.4 mg. adrenaline.

fastened it directly to a tension lever. It seemed possible that the depression they observed was due to the use of a very large dose. We found, however, that the injection of doses of adrenaline even as large as 1 mg. produced augmentation of the knee-jerk as shown in Fig. 5, and when the knee-jerk was abolished by the injection of 0.5 mg. nicotine tartrate, the subsequent injection of 0.4 mg. adrenaline restored it.

Using the perfusion system, we made observations on the flexor reflex also. When the blood flow through the cord was large, as for example in Fig. 6, the reflexes were small. The gradual addition of 0.1 mg. adrenaline

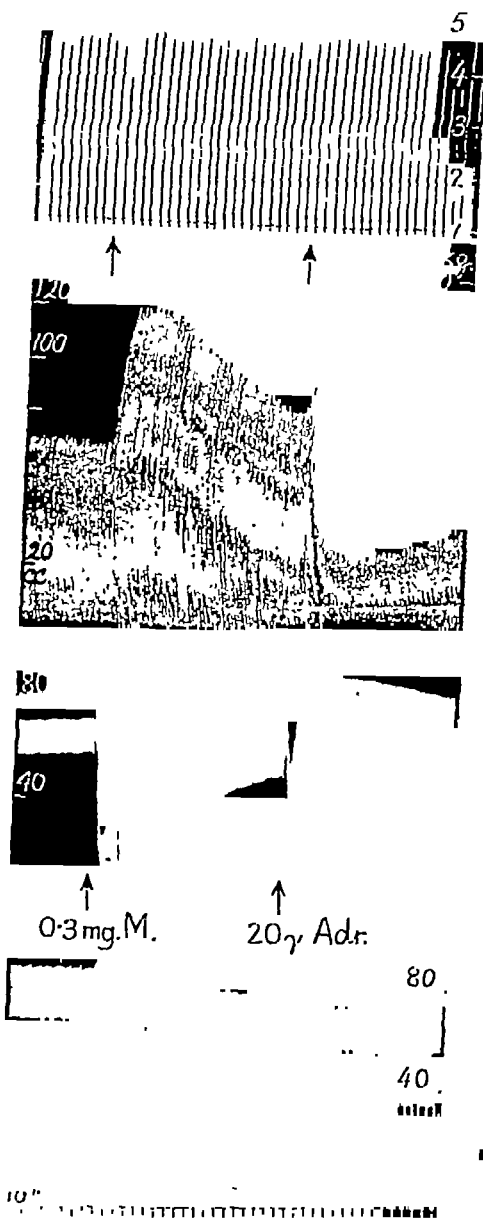


Fig. 3.

Fig. 3. Records as Fig. 2 except that top record is of the knee-jerk. The figure shows that the changes of blood flow produced by 0.3 mg. acetyl-β-methylcholine, and by 0.02 mg. adrenaline had no effect on the knee-jerk.

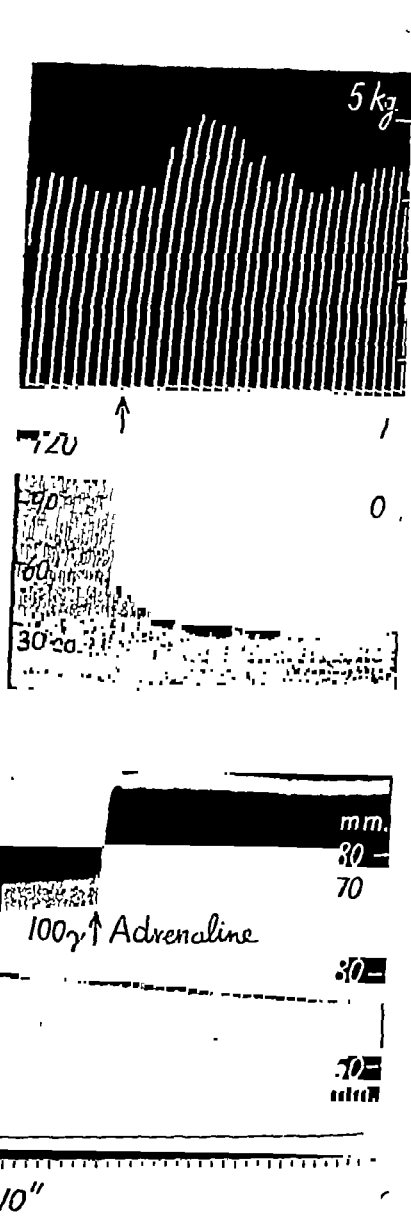


Fig. 4.

Fig. 4. Knee-jerk; other records as Fig. 2. Adrenaline, 0.1 mg., injected into cord circulation causing augmentation.

& Zeiss [1931] and Jacobsen & Kennard [1933]. We have found that both ephedrine and amphetamine increase the knee-jerk and the flexor reflex, and our records indicate that the effect on the knee-jerk is greater than that on the flexor reflex. This difference may be apparent rather than real, because the knee-jerk is more easily depressed by eserine and similar substances than is the flexor reflex, and we have usually injected ephedrine during a stage of depression. The augmentation produced by ephedrine was greater than that produced by amphetamine, but the two substances were not injected in similar doses; usually 1-2 mg. ephedrine sulphate was given and 0.1-0.5 mg. amphetamine. Larger doses of amphetamine caused persistent vasoconstriction in the cord circuit, and could not be used.

The action of eserine, prostigmine and nicotine on the knee-jerk and the flexor reflex

Schweitzer & Wright [1937 c, d, 1938] found that in cats under chloralose, eserine increased the knee-jerk, whereas prostigmine and nicotine depressed it. Merlis & Lawson [1939], however, found that in the dog eserine depressed the knee-jerk, though it augmented the flexor reflex. Bonnet & Bremer [1937b], working on the frog, examined the action of eserine on the reflex twitch of the semitendinosus provoked by stimulation of the homolateral sciatic nerve. They found that eserine augmented the after-discharge in the electromyogram and their records show that eserine diminished the height of the reflex twitch; there appears to be some contradiction between this observation and that of Merlis and Lawson on the flexor reflex. In agreement with Merlis & Lawson are McKail, Obrador & Wilson [1941] who found that eserine augmented the flexor reflex in the cat. Moreover, they found, as did Merlis & Lawson, that the action of eserine differed according to the process examined; thus when they stimulated the motor cortex or the pyramidal tract and recorded the contraction of tibialis anterior, eserine depressed the response.

Experimental results on the knee-jerk

Our own results with eserine, prostigmine and nicotine show that there is indeed a difference in the effect observed according to the reflex studied, but not according to the substance. As the observations of Schweitzer & Wright were the basis of the theory later put forward [Schweitzer, Stedman & Wright, 1939] to explain the difference between the action of eserine and prostigmine, we expected that eserine would increase the knee-jerk and carried out many experiments with a wide

during 13 min. to the reservoir from which the blood was taken to the cord augmented the reflexes to ten times their size. Discontinuance of the adrenaline led to a gradual decline of the reflexes, though the effect on them appeared to outlast that on the vessels.

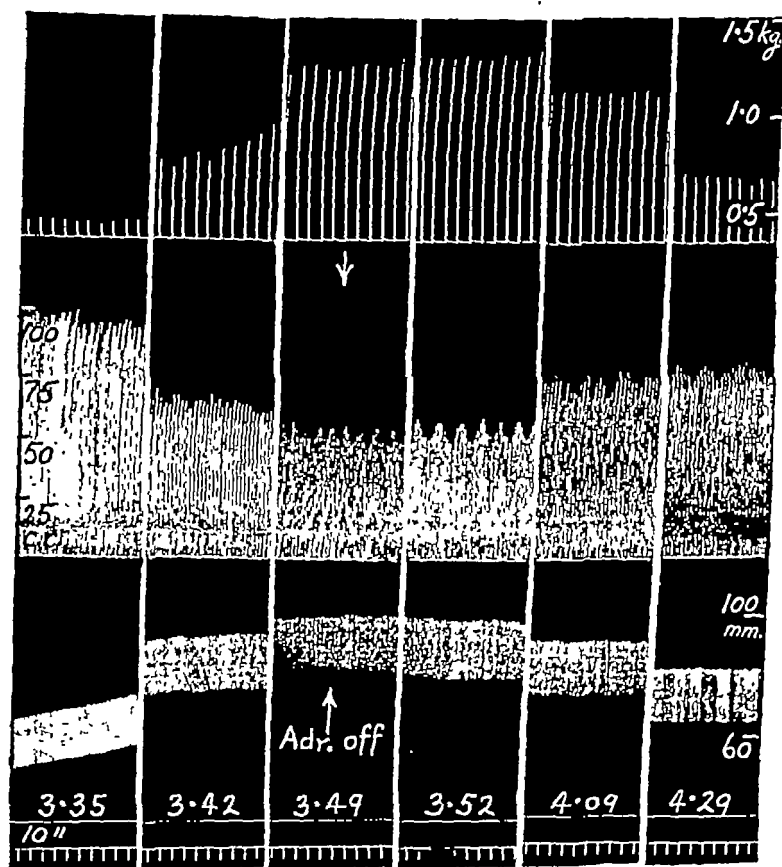


Fig. 6. Flexor reflex. Records from above downwards; contractions of m. tibialis anterior on stimulation of the posterior tibial nerve at intervals of 10 sec.; venous outflow (c.c./min.) from spinal cord; perfusion pressure in spinal cord. The flexor reflex is very weak when no adrenaline is present in the cord circulation. At 3.35 an adrenaline infusion was started, and 12 min. later it was stopped. Note the increased reflex response.

The action of ephedrine and amphetamine

Ephedrine, which would be expected to act in the same way as adrenaline, has been known to increase reflex excitability in dogs and monkeys since the work of Johnson & Luckhardt [1928], Hinsey, Ranson

Nicotine acid tartrate was effective in the same doses as eserine. The effects of prostigmine and nicotine are illustrated in Figs. 7*b* and 7*c*. These records are taken from different experiments. The evidence suggested in addition that the depressant action of nicotine was prolonged by the previous injection of eserine.

Experimental results on the flexor reflex

Merlis & Lawson [1939] have already shown that eserine increases the flexor reflex, and we have confirmed this observation as shown in Fig. 7*d*.

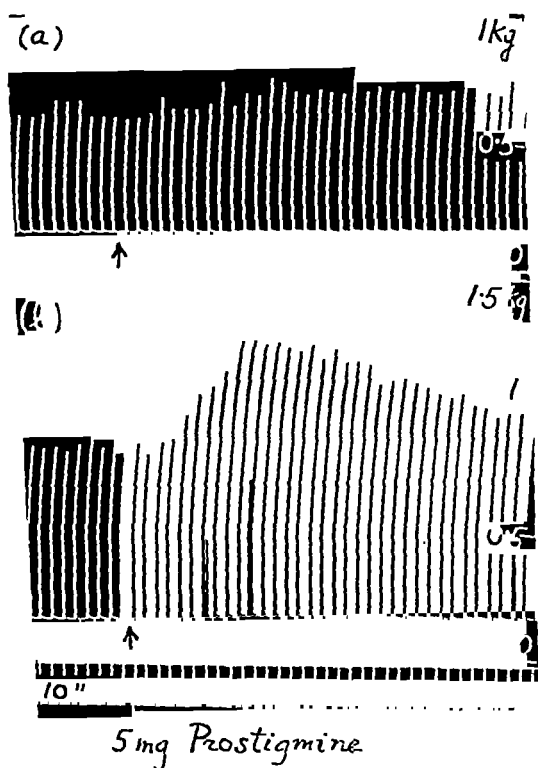


Fig. 8. Dog, double perfusion. Flexor reflex. (a) shows that 5 mg. prostigmine injected into the cord circulation scarcely modified the reflex response in the absence of adrenaline; (b) shows that after the addition of 0.1 mg. adrenaline to the cord circulation 5 mg. prostigmine augmented the flexor reflex.

This striking effect could be observed several times during an experiment, and even when the dose was increased to 5.0 mg., the effect was not

range of doses in the hope of observing this increase. The results were without exception in the opposite direction; the smallest dose which affected the knee-jerk at all diminished the tension as shown in Fig. 7a. This failure to observe an increase was not because the reflex tension was maximal, for it was possible to obtain an increase by injecting strychnine.

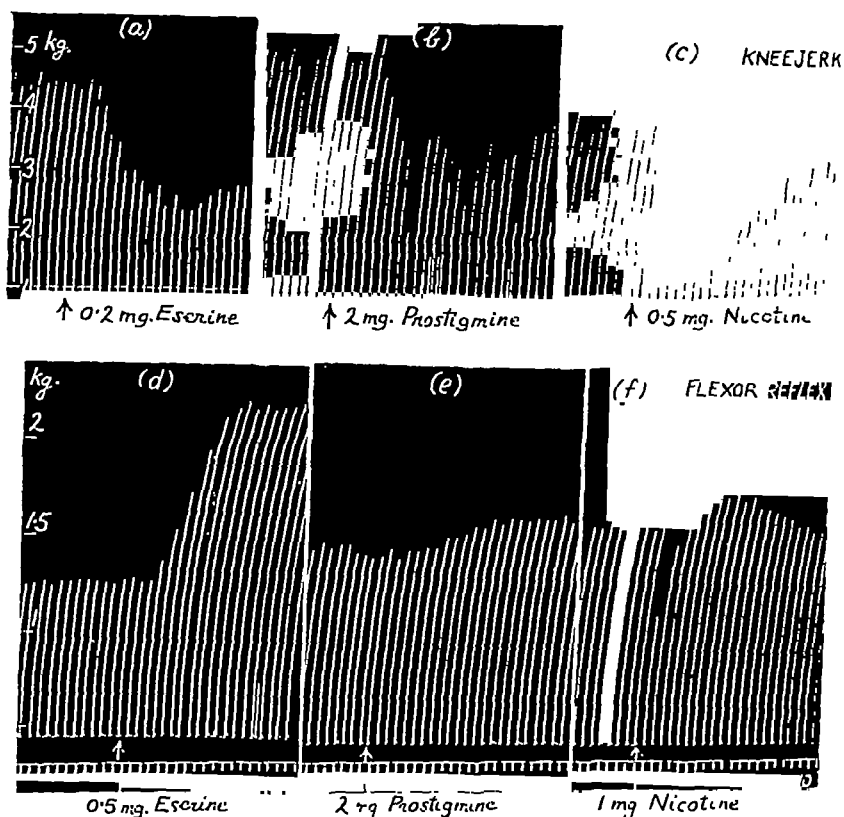


Fig. 7. Dog, double perfusion. Above, records of knee-jerk. Below records of flexor reflex. (a) and (d) show the effect of eserine; (b) and (e) show the effect of prostigmine; (c) and (f) show the effect of nicotine. All three substances depress the knee-jerk but augment the flexor reflex.

The action of prostigmine and nicotine on the knee-jerk was similar to that of eserine except that the depressant action of prostigmine was obtained with larger doses. Whereas eserine sulphate diminished the knee-jerk in doses from 0.2 to 0.5 mg. and abolished it in doses from 1.0 to 5.0 mg., prostigmine methyl sulphate diminished the knee-jerk in doses from 0.5 to 1.0 mg. and abolished it in doses from 2.0 to 5.0 mg.

think that the distinction between the action of acetylcholine before and after eserine is most important.

We have found that small amounts of acetylcholine up to $20\mu\text{g}$. injected into the cord circulation usually have no effect on the knee-jerk. Occasionally however, as shown in Fig. 9a, the knee-jerk was increased, the increase passing off in 1-2 min. Since this increase was accompanied by an increased blood flow, it may have been due to this increased flow. We do not think so because, as illustrated in Fig. 3, we have constantly observed that the knee-jerk remains unaffected by changes of flow.

When larger amounts of acetylcholine were injected, the first reflex after the injection was depressed (Fig. 9b) but even though the dose was

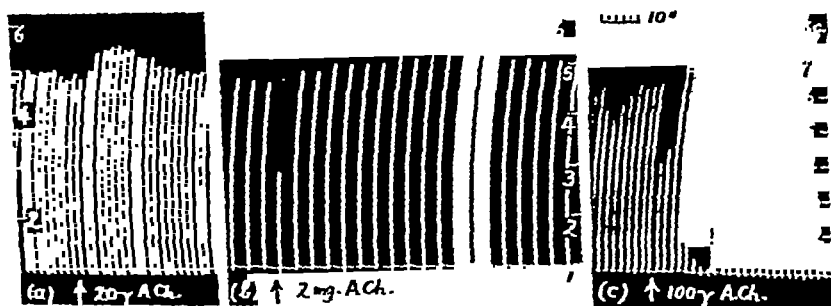


Fig. 9. Dog, double perfusion. Knee-jerk. (a) shows increased knee-jerk after the injection into the cord circulation of $20\mu\text{g}$. acetylcholine; (b) shows that 2 mg. acetylcholine diminished the first reflex following the injection and then augmented the knee-jerk; (c) shows that after eserine $100\mu\text{g}$. acetylcholine abolished the knee-jerk.

as large as 2.0 mg. this depression disappeared in 20 sec. and often gave place to a slight but maintained augmentation.

When eserine was injected beforehand, the effect of acetylcholine was different, and mainly depressant as shown in Fig. 9c. The actual sequence of changes was a short depression, a short augmentation and then a prolonged depression. This sequence is seen both in Fig. 14c in which the dose injected was $20\mu\text{g}$. and also in Fig. 9c when the larger dose of $100\mu\text{g}$. abolished the reflex. Thus the knee-jerk, which is depressed by eserine, prostigmine and nicotine, is likewise depressed by acetylcholine when this is injected after eserine or prostigmine.

On the flexor reflex the effect of acetylcholine was slight. Large doses such as 0.2 mg. caused a small increase in the reflex (Fig. 10a) which persisted for about 1 min. and which was not preceded by any trace of depression like that seen in Fig. 9b. The effect became more obvious after

The action of atropine

Langley & Kato [1915] observed that the central action of eserine was abolished by atropine. Schweitzer & Wright [1937 c] stated that atropine

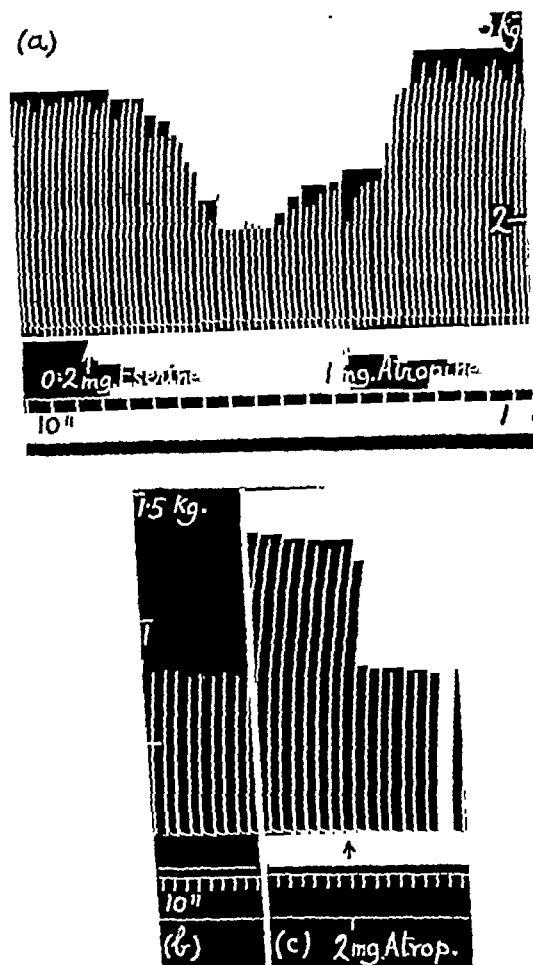


Fig. 12. Dog, double perfusion. Upper record: knee-jerk. Lower record, flexor reflex. (a) shows the depression of the knee-jerk by the injection into the cord circulation of 0.2 mg. eserine and the abolition of this effect by atropine; (b) shows the height of the flexor reflex before, and the increase (c) after the injection of 10 mg. prostigmine; atropine abolished the increase.

may itself diminish the knee-jerk; they could not find 'any direct pharmacological antagonism between eserine and atropine in their effects

the injection of eserine or prostigmine (Fig. 10*b*). There was a small initial depression, followed in 20–30 sec. by augmentation which lasted longer than the augmentation of the knee-jerk, and then a prolonged



Fig. 10. Dog, double perfusion. Flexor reflex. (a) Small increase produced by 200 μ g. acetylcholine injected into the cord circulation; (b) the effect of 100 μ g. acetylcholine after prostigmine. Note the difference between this effect and that in Fig. 9*c* where 100 μ g. acetylcholine abolished the knee-jerk.

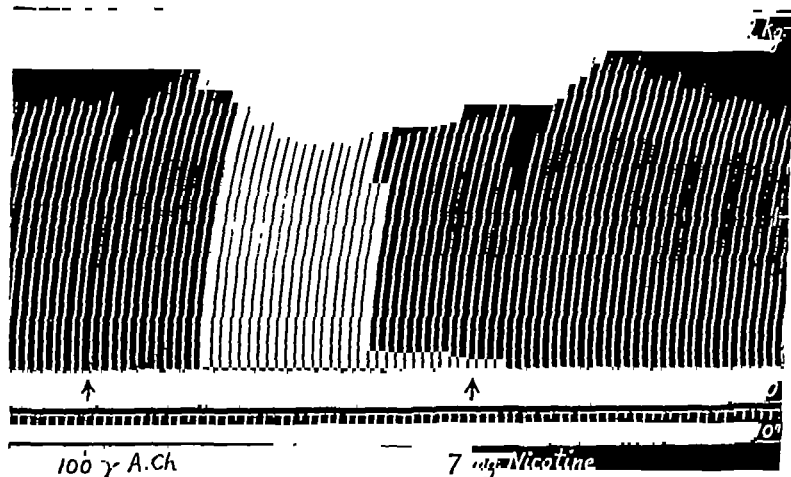


Fig. 11. Dog, double perfusion. Flexor reflex. The record shows the similarity of effects produced by 100 μ g. acetylcholine (after the injection of prostigmine) and 7 mg. nicotine.

though still slight depression. Just as eserine, prostigmine and nicotine even in large doses never depress or abolish the flexor reflex, so acetylcholine after eserine fails to depress it more than slightly. There was often a close resemblance between the action of acetylcholine after eserine or prostigmine and that of nicotine, as shown in Fig. 11.

The action of eserine on inhibition of reflexes

Torda [1940] has studied the crossed extensor reflex in the toad and its inhibition by stimulation of the sciatic nerve on the same side as that on which the reflex contraction was observed. She perfused the thoracic

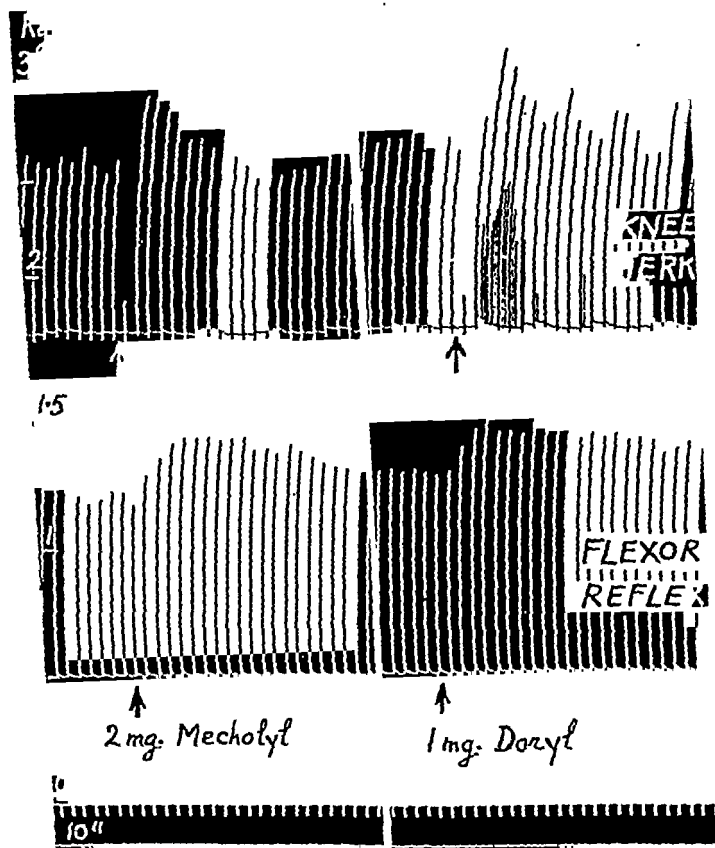


Fig. 13. Dog, double perfusion. Effects of acetyl- β -methylcholine and of carbaminoylcholine on knee-jerk and flexor reflex.

aorta with Ringer's solution, and added acetylcholine to the perfusing fluid. She found that acetylcholine depressed the crossed extensor reflex, but that the depressed reflex was not further depressed by stimulation of the homolateral sciatic nerve, but on the contrary was augmented, and that the augmentation outlasted the period of sciatic stimulation.

on reflex action', though the effects of atropine were in the reverse direction to those of eserine. Miller *et al.* [1940] found that atropine abolished the effects of acetylcholine and of eserine on the cortex to which reference has been made above. McKail *et al.* [1941] found that the effects of acetylcholine on the cortex, both before and after eserine, were abolished by atropine; further they found that the effects of eserine in depressing pyramidal stimulation and in augmenting the flexor reflex were also abolished by atropine.

Our results confirmed the antagonism between atropine on the one hand and eserine, prostigmine and acetylcholine on the other. After giving atropine, neither acetylcholine nor eserine were able to cause the discharge of impulses from the spinal cord; neither eserine nor prostigmine were able to depress the knee-jerk or to increase the flexor reflex. Further, as Fig. 12 shows, flexor reflexes augmented by prostigmine (or eserine) were reduced by atropine to their original size, and the knee-jerk depressed by eserine (or prostigmine) was promptly restored by atropine to its original size. Atropine by itself had no effect on the knee-jerk, and further it had no effect on the response to nicotine. The depression of the knee-jerk by nicotine recorded in Fig. 7c was obtained after giving atropine.

The action of stable choline esters

We have made a few observations only on acetyl- β -methylcholine and carbaminoylcholine and wish to make no more than a passing reference to them. We expected that the action of acetylcholine on the spinal cord would, like the action on sympathetic ganglia, be a 'nicotine' action [Dale, 1914], unaffected by atropine. We have seen that this is not so. We have also found that acetyl- β -methylcholine, which is devoid of nicotine action, affects both the knee-jerk and the flexor reflex, the effects on both reflexes being respectively similar to those of carbaminoylcholine. The knee-jerk is first inhibited and then augmented. The flexor reflex is simply augmented, this augmentation being more prolonged than that of the knee-jerk (see Fig. 13). We saw some indication that these immediate effects were followed by a 'long lasting progressive depression' of the reflexes similar to that observed in striated muscle by Bacq & Brown [1937], but more observations are necessary to establish this.

posterior tibial nerve was applied intermittently and alternately with the tap on the patellar tendon. In the absence of eserine such stimulation produced some depression of the knee-jerk, as shown in Fig. 15*a*, though not continuous depression. When the knee-jerk was depressed by eserine, the application of stimuli to the posterior tibial nerve augmented the knee-jerk as shown in Fig. 15*b*, the augmentation declining when the stimulation stopped. Sometimes the augmentation was seen only after the stimulation had stopped as in Fig. 15*c*.

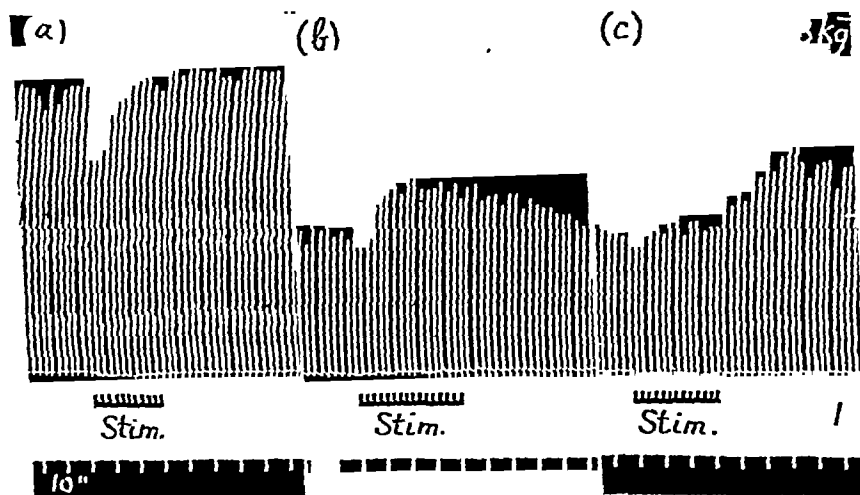


Fig. 15. Dog, double perfusion. Knee-jerk, elicited once in 3 sec. (a) shows the inhibition caused by stimulating the posterior tibial nerve for 1 sec. intermittently between the tendon taps; (b) shows the reversal of this inhibition after the injection of eserine; (c) shows augmentation appearing after the stimulation was stopped.

These effects of stimulation of the posterior tibial nerve on the knee-jerk have a similarity to the effects of injecting acetylcholine both before and after eserine. Thus Fig. 14*a* compares with Fig. 9*b*, in which the injection of a large dose of acetylcholine produced initial depression followed by slight augmentation. This augmentation or rebound of the knee-jerk after the inhibition produced by posterior tibial stimulation was seen sometimes but not always; similarly augmentation after the inhibition produced by acetylcholine injection was seen sometimes but not always. A further point of resemblance was that the depression following stimulation of the posterior tibial nerve could not be maintained by prolonged stimulation; it was greatest at first and had almost disappeared after 10 sec.; increase of strength of stimulus did not prolong it. The same

We have studied the inhibition of the knee-jerk when stimulation was applied to the posterior tibial nerve of the same side. In Fig. 14*a* is shown a record of the knee-jerk every 3 sec. and the depression of the tension caused by applying a tetanus to the posterior tibial nerve for 10 sec. The record shows that there was some augmentation of the knee-jerk for a period after the application of the stimulus, though this was not always seen. When however eserine was injected and the knee-jerk depressed to half its former size, as in Fig. 14*b*, stimulation of the posterior tibial nerve

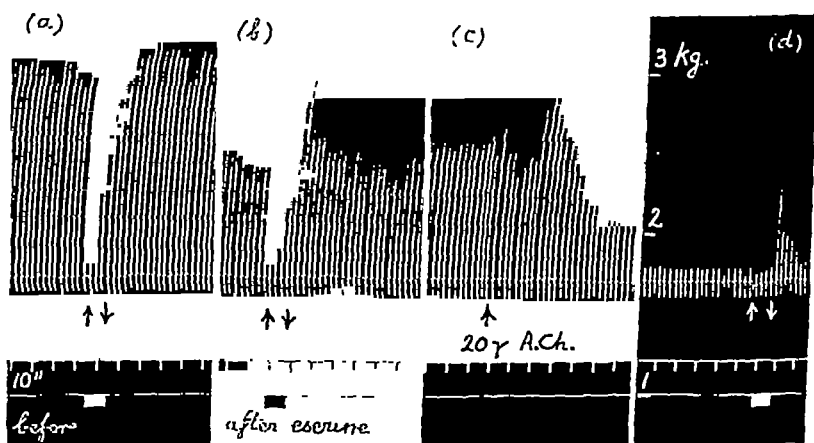


Fig. 14. Dog, double perfusion. Knee-jerk elicited every 3 sec. In (a) is shown the inhibition caused by a 10-sec. tetanus to the posterior tibial nerve. (b) shows the exaggerated rebound following the inhibition, when the reflex has been depressed by eserine. (c) shows a similar effect produced by the injection of 20 μ g. acetylcholine. (d) shows that when the knee-jerk is abolished by eserine, stimulation of the inhibitory nerve is followed by a reappearance of the reflex response.

still caused depression, but this was followed by a large rebound, so that the reflex tension was 40–50% greater than before. We never failed to observe this exaggerated rebound after eserine; it is perhaps most strikingly shown in Fig. 14*d*, in which the knee-jerk was actually abolished by eserine. (The tendon was still tapped at 3 sec. intervals, the marks on the record being made by the mechanical effect of the tapper.) A stimulus was then applied to the posterior tibial nerve for 10 sec. as shown, and when it stopped the knee-jerk suddenly reappeared giving a tension of 2.3 kg., though it declined again in 15 sec.

We have studied this inhibitory effect in still another way, which has given us results closely resembling those of Torda. The stimulation of the

SECTION III. LIBERATION OF ACETYLCHOLINE BY
NERVE STIMULATION

Various observers have described attempts to show that acetylcholine is liberated from the central nervous system when afferent nerves are stimulated [Dikshit, 1934; Feldberg & Schriever, 1936; Minz, 1936; Chang, Hsieh, Li & Lim, 1938; Li, 1938; Chute *et al.* 1940]. Mostly these results have been obtained on the brain, e.g. by stimulation of the vagus nerve, and few observations have been made on the spinal cord.

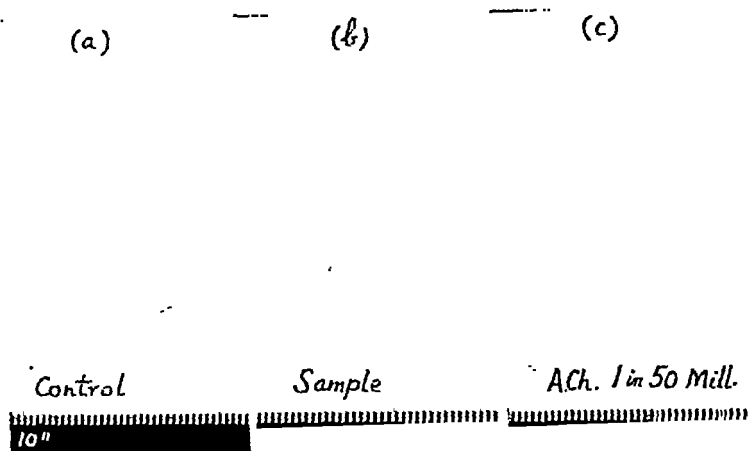


Fig. 17. Leech muscle (a) shows the effect of a control sample of venous effluent from spinal cord taken 3 min. after Ringer perfusion was started; (b) shows the contraction produced by a sample collected after 2 min. stimulation of the central end of the sciatic nerve; (c) shows the contraction produced by acetylcholine 1 in 50 million.

We first studied the length of time for which reflexes persisted when the cord circulation of the double perfusion system was changed from defibrinated blood to oxygenated and eserinated Ringer's solution. We found that the reflexes were initially augmented and then declined, but persisted for about 8 min. The reflexes were then only maintained if defibrinated blood was perfused once more. We made observations therefore by changing from a blood perfusion to a Ringer perfusion containing 1 in 400,000 eserine and 1 in 1 million adrenaline, and allowing 3 min. to elapse to wash the blood out of the system. Samples of venous effluent were then taken for control observations, and the central end of the cut sciatic nerve was stimulated with an induction coil (4 V. in the primary, secondary coil at 9 cm.). Samples of venous effluent were collected during 2 min. stimulation, and examined with the controls on the leech

was true of the injection of acetylcholine; no matter how large the dose, the initial depression was gone after 10–20 sec. After giving eserine the resemblance was still close. In Fig. 14c acetylcholine was injected when the knee-jerk was depressed; there was some further depression, a clear augmentation and then a further prolonged depression. This sequence is not wholly unlike that in Fig. 14b when the posterior tibial nerve was stimulated.

When depression was carried still further by the injection of nicotine as in Fig. 16, the chief effect of acetylcholine was to increase the knee-jerk, and the effect in Fig. 16a is strikingly similar to the effect of intermittent stimulation of the posterior tibial nerve shown in Fig. 16b.

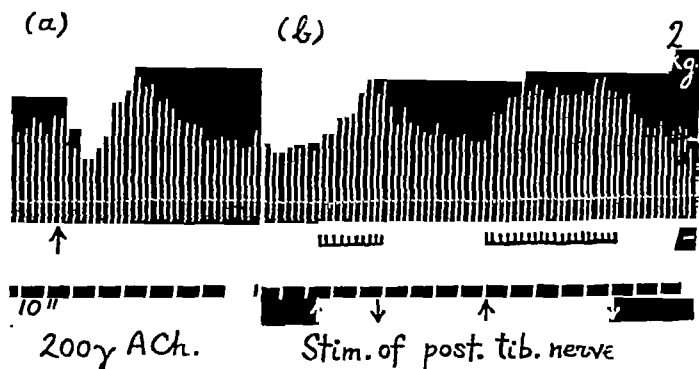


Fig. 16. Dog, double perfusion. Knee-jerk. (a) shows the reflex contraction depressed by 0.8 mg. nicotine; the injection of a large dose of acetylcholine temporarily augmented the knee-jerk; (b) shows similar augmentation caused by intermittent stimulation of the posterior tibial nerve.

When the knee-jerk was abolished by nicotine the injection of a large dose of acetylcholine restored it temporarily, just as did stimulation of the posterior tibial nerve as shown in Fig. 14d when the abolition was produced by eserine. It should be noted that these results are parallel to the results obtained by Bacq & Brown [1937] in striated muscle after giving nicotine. They found that intraarterial injection of nicotine caused muscular contraction, followed by depression of the response to single maximal shocks applied to the nerve. They say: 'This depression is remarkable in that it is removed completely by the stimulation of the nerve for a short time with a tetanizing current, and equally effectively by the arterial injection of sufficient acetylcholine.'

volving a larger number of neurones, and on the hypothesis of synaptic transmission by acetylcholine, a larger amount of that substance would be liberated.

When the action of drugs on the knee-jerk and the flexor reflex was examined, we found that the two reflexes behaved quite differently. The knee-jerk was readily depressed by eserine, prostigmine, nicotine and also by acetylcholine itself if eserine or prostigmine had been given previously. How are these observations to be explained if acetylcholine is the humoral transmitter? Should not eserine and prostigmine augment the knee-jerk by neutralizing the esterase? It may be recalled that in the sympathetic ganglion the usual effect of eserine is to depress the response to preganglionic stimulation and that an augmented response is only obtained when the stimulation is submaximal and set up at a very low frequency [Feldberg & Vartiainen, 1934]. In the flexor reflex, potentiation by eserine was readily observed and records were obtained closely resembling those showing the effect of eserine on the response of muscle to single maximal nerve shocks of low frequency [Brown, Dale & Feldberg, 1936]. We may, therefore, compare the reaction of the knee-jerk to the reaction of the ganglion, and that of the flexor reflex to the reaction of the neuromuscular junction. In our observations on the knee-jerk we have hitherto used a powerful tap chosen to give a large response in the quadriceps already under a tension of about 1 kg. It is conceivable that augmentation may be seen if a weaker stimulus is applied. Bacq & Brown [1937] showed that the effect of eserine on the neuromuscular junction depended on the frequency of stimulation; at frequencies above 24 per min. eserine depressed the response of muscle to nerve stimulation. It has yet to be determined what is the effect of eserine on a flexor reflex elicited at a higher rate than 6 per min. Further investigation may then reveal conditions in which eserine augments the knee-jerk and in which it depresses the flexor reflex. We do not think, therefore, that in the difference of the response of the knee-jerk and of the flexor reflex to the action of eserine as at present observed, there is any obstacle to the hypothesis of synaptic transmission by acetylcholine.

It has been advanced as an argument against synaptic transmission by acetylcholine that Bremer & Kleyntjens [1937], in studying the flexor reflex in the frog, did not find evidence that eserine altered the optimal interval for the summation of two submaximal stimuli, although they found that it increased the height of the reflex contraction. The curve relating height of contraction to the interval between stimuli was not lengthened as would have been expected if the summation was actually

muscle. Fig. 17 shows that the venous effluent collected during stimulation was equivalent in activity to 1 in 50 million acetylcholine, while the control sample was equivalent to 1 in 1000 million. Several experiments were performed each of which gave a positive result. Since it was striated muscle surrounding the vertebral column in the circulation, we cannot say that the acetylcholine liberated certainly from the spinal cord, and more experiments are required to establish

DISCUSSION

We have shown that when so small a quantity of acetylcholine $1\mu\text{g.}$ was injected into the circulation through the spinal cord, a discharge of motor impulses occurred which was recorded as a series of contractions of the tibialis anterior. It is possible that this discharge was due to a lowering of the threshold of the efferent path of the reflex arc to impulses otherwise imperceptible, arriving along the afferent path; this we have not yet excluded. We think, however, that this action of acetylcholine is a direct stimulation of the motor neurones similar to that produced by doses of eserine, prostigmine or nicotine 1000 times greater.

The action of acetylcholine was facilitated and indeed almost dependent on the presence of adrenaline in the circulation. The effect of adrenaline is probably similar to its action at the neuromuscular junction where it improves the transmission once this has been diminished by fatigue; we think of this action in the spinal cord as a lowering of the threshold for stimuli reaching the motor cell, for we found that adrenaline had a similar facilitating action on reflexes, in particular on the flexor reflex.

This relation between the action of adrenaline and acetylcholine may explain the results obtained by Bonvallet & Minz [1938] who describe a parallelism between the action of acetylcholine and that of adrenaline on spinal reflexes in various animals; they found that the effect of adrenaline, however, was produced after a longer latent period, but that the effect of both substances was abolished by atropine. Now Feldberg & Schrier [1936] found that in the eserinated cat, the intravenous infusion of adrenaline led to an increase in the acetylcholine present in the cerebrospinal fluid. Bonvallet and Minz therefore conclude that the evidence indicates that adrenaline actually liberates acetylcholine within the spinal cord, and that all these results are to be explained in this way. We think, however, that there is another explanation. If adrenaline lowers the threshold for the transmission of impulses within the cord, its administration will result in an increased spread of a given sensory impulse, i

necessary to produce various effects in the frog (inhibition of the heart, contraction of rectus abdominis) and the amount of atropine necessary to abolish these effects.

Very large amounts of acetylcholine can be injected without having more than a transient effect on reflexes. It is essential for the hypothesis of acetylcholine transmission that the spinal cord should be able to remove or inactivate very large amounts of the transmitter in the shortest time. If however the removal process is blocked by the injection of an anticholinesterase the situation is completely altered, and acetylcholine has then the same effect on the reflexes which the anticholinesterases have themselves. Like eserine, prostigmine and nicotine, it then depresses or abolishes the knee-jerk; on the flexor reflex, like these substances, acetylcholine has scarcely any depressor and sometimes it has an augmentor action.

The fact that acetylcholine after eserine does not abolish the flexor reflex seems to us to present difficulty in interpretation. We know that at the motor end plate in skeletal muscle, Bacq and Brown observed that large doses of eserine themselves have no depressant action provided that the rate of stimulation is slow; they did find however that in the presence of eserine, acetylcholine caused depression. Why does this not occur in the flexor reflex as it does in the knee-jerk? Whatever be the explanation, it is clear that the flexor reflex is a mechanism extremely resistant to depression.

In a final comment on the action of drugs we wish to call special attention to the inhibition of the knee-jerk by stimulation of the posterior tibial nerve, and to the effect of eserine upon it. If the knee-jerk is depressed by eserine, the rebound of the knee-jerk when the inhibitory stimulus stops becomes greatly exaggerated; if the knee-jerk is abolished by eserine the inhibitory stimulus then actually restores the knee-jerk. The interesting point about these results is that at all stages the effect of the inhibitory stimulus can be imitated by an injection of acetylcholine.

When the knee-jerk is abolished by nicotine, it is temporarily restored by the injection of a large dose of acetylcholine. We have then two contradictory effects of acetylcholine; after eserine or prostigmine have been added to the circulation, the injection of acetylcholine depresses the knee-jerk, or even abolishes it. On the other hand, when the knee-jerk is abolished by nicotine the injection of acetylcholine temporarily restores it. It is surely significant that the same contradiction was observed by Bacq and Brown in striated muscle. After the injection of 100 μ g.

based on the persistence of the acetylcholine liberated by the first impulse. We feel that this argument loses much of its force in view of the fact that Bremer & Kleyntjens were working on the frog. We find it difficult to suppose that in cold-blooded animals the anticholinesterase action of eserine has the same importance as in warm-blooded animals. For example, Feng [quoted by Brown, 1937] has observed that the eserine potentiation of the muscle response to nerve stimulation in frogs is observed only if the interval between the stimuli is as long as 5 min.; otherwise it is absent.

A subsidiary problem which has arisen concerns the difference in the action of the substances eserine and prostigmine. In the cat under chloralose Schweitzer & Wright [1937 c, d] found that eserine augmented, and prostigmine depressed the knee-jerk; in support of this difference in the central action of these two substances are the observations of Williams & Russell [1941] on epileptics, who found that whereas prostigmine caused an increase in petit mal activity, recorded in the electrocorticogram, eserine reduced this activity, though with large doses the activity was also increased by eserine. We ourselves found no difference whatever in the action of eserine and prostigmine on the knee-jerk; both were equally depressant. Similarly, on the flexor reflex the action of both was in the same direction; we found, however, that eserine had much more potentiating influence than prostigmine, irrespective of the dose used; that is to say even 5 mg. prostigmine had less augmentor effect on the reflex than 0.5 mg. eserine. Potentiation by eserine was rapid, whereas that due to prostigmine was slow. Our results indicated that the addition of adrenaline altered the effect of prostigmine so as to resemble more closely that of eserine; perhaps this may be explained by some alteration of cell permeability whereby prostigmine, which may be otherwise a less diffusible substance than eserine, approaches nearer to it in intensity of action. Schweitzer, Stedman & Wright [1939] suggest that eserine diffuses into and acts within the cell, whereas prostigmine cannot enter and acts outside. We should emphasize, however, that between eserine, prostigmine and nicotine we have observed no fundamental difference in the direction of their action.

We observed that atropine completely prevented (if given before) or removed (if given after) the effects of acetylcholine, eserine and prostigmine. In a recent paper Abdon [1940] has shown that it is untrue to suppose that the 'nicotine' actions of acetylcholine are unaffected by atropine since large doses of atropine do in fact abolish them; he finds that there is a constant ratio between the amount of acetylcholine

7. The inhibition of the knee-jerk by stimulation of the posterior tibial nerve has been studied. After giving eserine, the inhibition is followed by an exaggerated rebound, and if the inhibitory stimulus is alternated with the tap on the patellar tendon, the knee-jerk is augmented. If the knee-jerk is abolished by eserine or nicotine and the posterior tibial nerve is stimulated, the knee-jerk is temporarily restored. All these effects of posterior tibial nerve stimulation are comparable with the effects of injecting acetylcholine, both before and after eserine.

8. If the blood in the cord circulation is replaced by Ringer's solution containing eserine, the reflexes persist for about 8 min. If during this period the central end of the cut sciatic nerve is stimulated, acetylcholine appears in the venous effluent in a concentration of 1 in 50 million.

9. The discharge of impulses from the spinal cord following the injection of acetylcholine is greatly facilitated by the presence of adrenaline. Similarly, in this double perfusion system the flexor reflex sometimes becomes very weak; it is then restored by the addition of adrenaline to the perfusing blood. Sometimes adrenaline augments the knee-jerk; we have never observed adrenaline to depress it.

We wish to place on record the very great help we received from Mr H. W. Ling in carrying out these experiments.

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miotine they found that the arterial injection of $10\mu\text{g}$. acetylcholine caused a depression of the contractions of the gastrocnemius in response to maximal shocks to the nerve. On the other hand they were able to depress the muscle response by an injection of nicotine, and then found that this depression could be removed by the injection of acetylcholine.

We have shown that acetylcholine is liberated in the cord circulation when the central end of the sciatic nerve is stimulated; these experiments are of course preliminary since it is essential to exclude the paravertebral muscles as the source of this acetylcholine. Nevertheless the observation brings support to our general conclusion that every test which we have so far applied to the perfused spinal cord has given a result which is consistent with synaptic transmission by acetylcholine and which corresponds to a result obtained either at the neuromuscular junction or at the sympathetic ganglion.

SUMMARY

1. A method is described of perfusing the lower half of the spinal cord of a dog, and of perfusing by a second circulation the muscles of the hindleg. By means of this preparation the effect of drugs applied to the spinal cord on reflex contractions of the leg muscles can be studied.

2. We have found that when acetylcholine is injected into the spinal cord (when no reflexes are being elicited) a discharge of motor impulses occurs when the dose is as small as $1\mu\text{g}$.

3. Acetylcholine injected into the cord circulation in the largest doses has only a very transient depressant effect upon the knee-jerk, often followed by some augmentation. On the flexor reflex it produces slight augmentation. These findings apply to observations made before the injection of any anticholinesterase.

4. Eserine, prostigmine and nicotine all depress the knee-jerk, and all augment the flexor reflex; the augmentor effect of nicotine on the flexor reflex is always preceded by transient depression. On the flexor reflex the augmentor action of eserine is much greater than that of the other two substances. The augmentor action of prostigmine is increased by the presence of adrenaline.

5. After eserine or prostigmine, acetylcholine depresses or abolishes the knee-jerk; on the flexor reflex it produces a very slight depression, but this is preceded by phases of transient depression and augmentation. The action of acetylcholine in the presence of eserine or prostigmine is very similar to that of nicotine both on the knee-jerk and on the flexor reflex.

6. Atropine prevents or abolishes all actions of acetylcholine, eserine and prostigmine on the spinal cord.

7. The inhibition of the knee-jerk by stimulation of the posterior tibial nerve has been studied. After giving eserine, the inhibition is followed by an exaggerated rebound, and if the inhibitory stimulus is alternated with the tap on the patellar tendon, the knee-jerk is augmented. If the knee-jerk is abolished by eserine or nicotine and the posterior tibial nerve is stimulated, the knee-jerk is temporarily restored. All these effects of posterior tibial nerve stimulation are comparable with the effects of injecting acetylcholine, both before and after eserine.

8. If the blood in the cord circulation is replaced by Ringer's solution containing eserine, the reflexes persist for about 8 min. If during this period the central end of the cut sciatic nerve is stimulated, acetylcholine appears in the venous effluent in a concentration of 1 in 50 million.

9. The discharge of impulses from the spinal cord following the injection of acetylcholine is greatly facilitated by the presence of adrenaline. Similarly, in this double perfusion system the flexor reflex sometimes becomes very weak; it is then restored by the addition of adrenaline to the perfusing blood. Sometimes adrenaline augments the knee-jerk; we have never observed adrenaline to depress it.

We wish to place on record the very great help we received from Mr H. W. Ling in carrying out these experiments.

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miotine they found that the arterial injection of $10\mu\text{g}$. acetylcholine caused a depression of the contractions of the gastrocnemius in response to maximal shocks to the nerve. On the other hand they were able to depress the muscle response by an injection of nicotine, and then found that this depression could be removed by the injection of acetylcholine.

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6. Atropine prevents or abolishes all actions of acetylcholine, eserine and prostigmine on the spinal cord.

A NOTE ON INTERACTION BETWEEN
NERVE FIBRES

BY BERNHARD KATZ

*Kanematsu Memorial Institute of Pathology,
Sydney Hospital, Sydney, Australia*

AND OTTO H. SCHMITT

*Departments of Physics and Zoology, University of Minnesota,
Minneapolis, Minnesota, U.S.A.*

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It has previously been shown [see Katz & Schmitt, 1939, 1940 for details of problem and methods] that the passage of an impulse in a single nerve fibre of *Carcinus* (fibre I) is accompanied by a triphasic excitability change consisting successively of a fall, a rise, and a second fall of excitability in an adjacent fibre (fibre II). These changes were attributed to that part of the action current which penetrates the resting fibre and which reverses in direction twice as does the excitability change. Recently, Blair & Erlanger [1940] have raised the question whether those changes may not, to some extent, have been due to a resistance decrease of the active fibre [cf. Cole & Curtis, 1939]. It is conceivable, as they point out, that the active region of fibre I may provide a more effective shunt for the test stimulus and so cause an apparent fall in excitability of fibre II, possibly masking or preceding an excitability rise due to the action current.

In the case of two *Carcinus* fibres the question can be decided as follows. Consider the arrangement of Fig. 1*a*. The test shock is applied at *C* and *D*, with cathode at *D*. A depression of excitability due to more effective shunting would occur as soon as the impulse reached the inter-polar stretch *CD*. The latency of this effect, therefore, depends upon the position of the anode *C*. On the other hand, changes due to penetrating action currents occur only when the impulse reaches the cathode *D* and are independent of the position of the anode.

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opposite phase of threshold lowering: a much smaller initial rise of threshold would, therefore, be expected in Fig. 1*b*. These expectations, however, are not borne out by experiment (Fig. 1 and Table 1). The excitability changes remain essentially unaltered after reversal of electrodes *C* and *E*. The first phase, while delayed by about 0.1 msec., is actually somewhat larger than in Fig. 1*a*. The small delay must, in part at least, be attributed to the fact that, by reversal of electrodes *C* and *E*, the position of the cathode is moved effectively across the width of the contact *D* (150 μ wire+droplet of saline giving a width of 0.2–0.3 mm.). It appears, therefore, that the impedance change in the active fibre is not large enough to alter appreciably the efficacy of the test stimulus and that transverse action currents, as previously explained, are mainly or solely responsible for the observed excitability changes.

SUMMARY

Further evidence is brought forward to show that the passage of an impulse in a single nerve fibre of *Carcinus* is accompanied by a genuine change of excitability in an adjacent fibre. The suggestion of Blair & Erlanger [1940] that the apparent change of excitability might be due to a change of resistance in the active fibre will not account for the observations here described.

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Thus it is possible to distinguish between the two effects by interchanging the positions of electrodes *C* and *E* (anode and grid respectively). If the threshold rise in Fig. 1*a* were largely due to a shunting

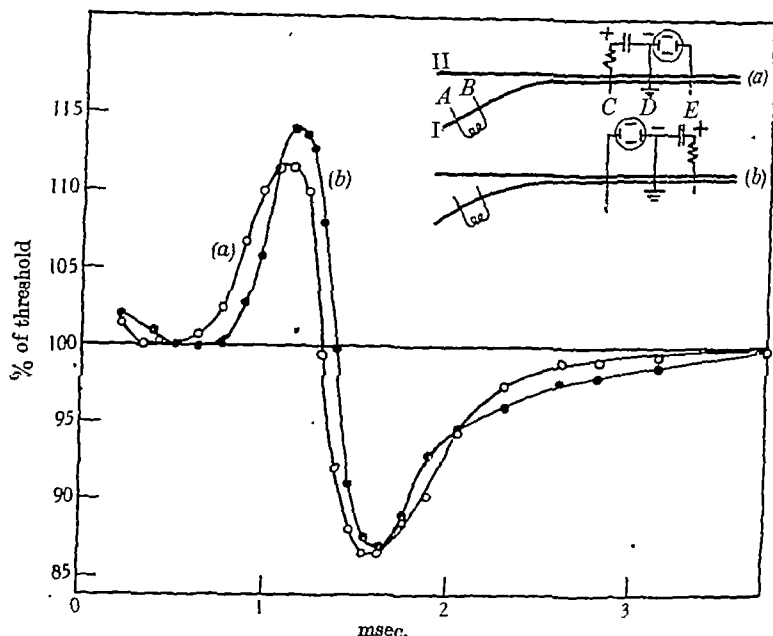


Fig. 1. Excitability changes in fibre II. Two *Carcinus* axons, at 19.5° C. Inset showing electrode arrangement. I and II, active and test fibre respectively. *AB*, stimulating leads to fibre I, *CDE* leads for test stimulus (cathode at *D*) and recording (earth at *D*). (a) and (b) are identical except that positions of test anode and grid lead (*C* and *E*) are interchanged. Distances *CD* and *DE* about 3 mm. Ordinates: threshold strength of test shock in percentage of normal. Abscissae: time interval between conditioning shock (applied at *AB*) and test shock, in msec. The small change during the first $\frac{1}{2}$ msec. is due to interaction between the two stimulating circuits. This is followed by a diphasic excitability change; the third phase previously described was not observed in this case [cf. Katz & Schmitt, 1940, p. 475].

TABLE 1

	Peak time after first shock in msec.		Peak size in percent of normal threshold	
	(a)	(b)	(a)	(b)
Threshold rise	1.08	1.13	112	114
Threshold fall	1.61	1.63	86.5	87

effect, then a major part of it should be delayed by at least 0.5 msec. (minimum conduction time for distance *CD*) in the case of Fig. 1*b*. As a consequence there would be an increased interference with the

weaning (21 days) on the same basal diet to which Ca lactate (B.P.) and Na_2HPO_4 (A.R.) had been added to give a Ca/P ratio of 1. The diet given to one group of rats (Group I, Nos. 1, 2, 3 and 4) contained 0.30% of both Ca and P, whilst the diet given to the other group of rats (Group II, Nos. 5, 6, 7 and 8) contained 0.12% of each of these elements. The construction of the basal diet, which contained egg albumin, wheat gluten, corn starch, butter fat, a salt mixture and an inorganic phosphorus-free extract of yeast, has been described in full in a previous paper [Gaunt & Irving, 1940]. In the experiments described in that paper, it was found that, whilst the diet containing the higher amounts of Ca and P induced normal calcification of the teeth and slightly subnormal calcification of the bones, the diet containing the smaller amounts of these elements interfered markedly with the calcification of the skeleton, judged chemically, and of the teeth, as judged histologically.

The rats were housed in individual steel cages in the same room at a temperature of 68–70° F. and were given the diets and distilled water ad lib. Twenty-eight days later, when they were 49 days old, they received subcutaneous injections of a solution containing radio-P. The sample of radio-P was contaminated when received with iron salts and other material, and was purified by successive precipitations as ammonium phosphomolybdate and magnesium ammonium phosphate. The final precipitate of magnesium ammonium phosphate was dissolved in water and the solution evaporated to dryness. The residue was taken up in 50 c.c. distilled water and aliquots of this solution, varying between 4.70 and 5.91 c.c., taken in proportion to the weights of the rats, were pipetted into 25 c.c. beakers and evaporated to dryness. A ninth aliquot for use as a standard was treated in the same way. Each of the dried portions was dissolved in 0.8 c.c. hot water containing 0.25% phenol and the solutions when cool were injected subcutaneously into the rats in the order of increasing weights using the same syringe throughout. The standard solution was also taken up in the syringe and diluted to 10 c.c. with distilled water. The total P content of the injections was negligible compared to the P content of the rats.

Immediately after injection the rats were transferred to stainless steel metabolic cages of the Hopkins type, the same diets being continued. The faeces and urine were collected separately. The rats were killed by coal-gas 90 hr. after the injections and dissected into the following fractions: skin, muscles, liver, kidneys, molar teeth, incisor teeth, skeleton and brain. The remaining organs, i.e. the lungs, heart, stomach, intestines, reproductive organs, etc., were all taken together and labelled 'viscera'.

THE ASSIMILATION OF RADIO-ACTIVE PHOSPHORUS FOLLOWING PHOSPHORUS DEFICIENCY IN RATS

By W. E. GAUNT, H. D. GRIFFITH AND J. T. IRVING

From the Rowett Research Institute, Aberdeen, the Natural Philosophy Department, University of Aberdeen, and the Department of Physiology, University of Cape Town

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THE use of radio-active isotopes as indicators of mineral metabolism was begun in 1923 when Hevesy investigated the uptake of radio-lead by plants. The recent production of radio-phosphorus has made this isotope also available for metabolic studies. Chievitz & Hevesy [1935], Hevesy [1938, 1939], Artom, Sarzana, Perrier, Santangelo & Segré [1937] and others have shown that, after administration to animals, all the tissues immediately take up radio-P in varying amounts, the distribution in the body altering slowly with time.

The effect of changes in P metabolism upon the distribution of radio-P has also been investigated. Chievitz & Hevesy [1937], Dols, Jansen, Sizoo & de Vries [1937], Dols, Jansen, Sizoo & van der Maas [1938b] and Cohn & Greenberg [1939] have reported the findings with rachitic animals, both before and after vitamin D medication. However, no investigation appears to have been made of the changes in the detailed distribution of radio-P in the body when given to animals after subjection to mild P deprivation for several weeks. The following experiments were undertaken to investigate whether such a regime would affect the uptake of radio-P by different tissues of the body.

METHODS

The experiment was conducted on a single litter of four male and four female albino rats. The litter was divided into two groups each containing two males and two females. Both groups were fed from

small tinned iron capsules and bound together with a few drops of a dilute solution of collodion in ether.

Standards of magnesium ammonium phosphate containing known amounts of the radio-P solution were also prepared. After the amount of P present in the standard solution of radio-P had been determined, 5 c.c. of this solution was diluted to 100 c.c. Aliquots ranging from 20 to 0.16 c.c. of the dilute radio-P solution were taken and to each of them 15 c.c. of 1% Na_2HPO_4 solution were added. The P in these various standards was precipitated by the method described above and 100 mg. samples of the dried magnesium ammonium phosphate precipitates were weighed into iron capsules and bound with collodion.

After all the samples had been examined for radio-activity the phosphorus content of some of the magnesium ammonium phosphate precipitates was determined. It had been hoped that the composition of these precipitates would be constant, but this was not the case. Determinations of the P content of each 100 mg. sample were therefore made in the following way. The samples were transferred from the iron capsules to silica basins by washing first with ether and then with dilute HCl. The ether was removed on the water bath and the aqueous solutions evaporated to dryness. The residues were treated with dilute HCl, transferred to 100 c.c. graduated flasks and P determinations made in duplicate of aliquots of each solution. The small amount of collodion present was removed by filtration when necessary, and in any case did not interfere with these determinations.

Counting equipment. The counters employed were constructed from Pyrex tube, internal diameter 20 mm., and were about 15 cm. in length overall (Fig. 1). Each had a thin window blown at the side, where the wall thickness was not greater than 0.1 mm.; this came opposite a thin window of aluminium foil covering a hole in the copper cathode of the counter, to admit β -radiation from outside with minimum loss. The anode of the counter was a tungsten wire, diameter 50 μ , stretching along the axis.

Using a mixture of argon at a pressure of 90 mm. mercury with alcohol vapour at 15 mm. as the filling gas [Trost, 1938], the counters were found to have a satisfactory 'plateau' where counting rate was sensibly independent of voltage (within $\pm 2\%$) over a range of 850-920 V. The spontaneous count was very constant at about 30 discharges per min.: this rather large value was considered inevitable in apparatus constructed in the laboratory of a radon and radium centre.

The high-tension supply was drawn from a stabilized rectifier of conventional design giving voltages up to 1200 V., and the pulses discharged from the counter were fed into a two-stage resistance-capacity coupled amplifier, which actuated a thyratron. The pulses were then registered on a standard telephone call counter, one of the contacts of which had been reversed so as to interrupt the anode circuit of the thyratron to 'quench' it.

The counter was mounted so as to be completely screened by 2.5 cm. of lead in all directions except for a hole 1 cm. in diameter opposite to the window for the entrance of

The separation of the different fractions was made as distinct as possible, the bones being cleaned mechanically by scraping with a scalpel. The scrapings were added to the muscle fraction. Only the paws were rejected.

The right femur, tibia and fibula of each rat was separated from the skeleton, extracted with boiling alcohol and then with ether and dried at 100°. They were then weighed and the ash content determined by heating them in silica crucibles at 650° till they had constant weight. The incisors and the remaining bones were dried at 100°, weighed and the ash content also determined. The molars were ashed without previous drying and weighing. The other fractions, including the faeces, were placed in silica basins and were thoroughly mixed with a little hot 20% calcium acetate solution to ensure against the loss of P during the subsequent ashing and drying. It was not found practicable to weigh these fractions before ashing. The urine samples were also treated with Ca acetate and evaporated to dryness in silica basins. In all cases the organic material was burnt away, the ashing being completed in a muffle furnace.

The ash of each sample was extracted from the silica basins with boiling concentrated HCl and the extracts transferred to graduated flasks. To some of the ash solutions an accurately measured quantity of a 1% solution of Na_2HPO_4 was added before being diluted to the mark with water. Ten c.c. of the Na_2HPO_4 solution were added to the skin, kidney and brain ash solutions and 5 c.c. to the liver, molar and incisor ash solutions. No additions of phosphate solution were made at this stage to the other ash solutions.

Phosphorus determinations were carried out in duplicate on each of the ash solutions by the method of Fiske & Subbarow [1925]. From the remainder of each solution the Ca was precipitated completely by McCrudden's method [1909-10]. The Ca oxalate was filtered off and magnesium ammonium phosphate was precipitated from the filtrate by the addition of a large excess of magnesium citrate mixture. Some of the urine ash and faeces ash solutions contained only small amounts of P, and additions of known amounts of the standard phosphate solution were made to these ash solutions before the removal of the calcium and the precipitation of the P as magnesium ammonium phosphate.

After standing for at least 12 hr. the crystalline precipitate of magnesium ammonium phosphate obtained from each sample was filtered off, washed well with cold dilute ammonia solution and then dried at 100°. Portions of 100 mg., or as near 100 mg. as possible, were weighed into

gave the activity of the material under study. The following may be quoted as a typical set of observations:

Spontaneous discharge rate	32 per min.
Standard capsule S5 ...	activity 0.105 c.c. stock solution
S5 under counter ...	1207 counts in 5 min.
	or 241 counts per min.
Less spontaneous count ...	209 counts per min.
Capsule 41 under counter	1660 counts in 7½ min.
	or 222 counts per min.
Less spontaneous count	190 counts per min.
Hence activity of capsule 41	$= 0.105 \times \frac{190}{209}$
	$= 0.096$ c.c. stock solution

The technique employed had several advantages. Since it involved the use of the counter as a transfer instrument, for comparison of an unknown material with a closely similar standard, many difficulties of design met with when counters are employed as absolute meters were avoided.

The writers consider that they have succeeded in compensating for the partial absorption of β -radiation coming from deeper layers of the active material, which act as a screen during measurement, by using 100 mg. of active material in every case spread uniformly in capsules of standard area. The active material was in every case a phosphate precipitate, unmixed with ash or other diluent, the light powdery phosphate being bound only by addition of a small standard amount of a weak solution of collodion in ether to ensure against accidental losses in transport.

The decay of the active material affected both standard and unknown equally and therefore did not enter into the computation of the activity of a sample. Decay during the progress of a run of the counter lasting a few hours was inappreciable within the limits of error of animal experiment.

The accuracy of the technique adopted for the analysis of the radio-P content of the different tissue samples is well illustrated by Table 1.

TABLE 1. Percentage recovery of radio-active P

Rat no.	Vol. radio-P solution injected c.c.	Vol. radio-P solution recovered c.c.	% radio-P recovered
1	5.91	5.853	99.1
2	5.85	5.548	95.5
3	4.86	5.165	106.0
4	4.87	4.868	100.0
5	4.83	4.776	98.7
6	5.86	6.321	107.9
7	4.70	4.387	93.4
8	4.78	4.750	99.4

Despite the many processes through which the eleven samples from each rat were put, the recovery of radio-P varied between 93 and 108%, and in five instances was almost exactly 100%.

the β -radiation. Since the counters were definitely photo-sensitive, the screens were designed to exclude light from the tube as well as to absorb stray radiation of more penetrating types as far as possible. The tin capsules containing the radio-P precipitates could be introduced on a simple sliding holder into standard positions opposite the window and either 2 or 5 cm. from the axis of the counter. The larger distance was convenient for some of the more active preparations which would give inconveniently high counting rates if placed too near the window.

Technique of counting. Observation of the spontaneous discharge rate was first made over a period of some 10 min. with an inactive capsule in the slider. A standard capsule, containing 100 mg. of phosphate precipitate including radio-P from a known volume of the stock solution, was then put below the counter and the discharge rate observed over a time sufficient to record at least 900 impulses. Similar observations were then made with the other standard capsules, the stronger ones being placed at the greater of the two standard distances from the counter, so as to avoid counting rates above 250 impulses per min.

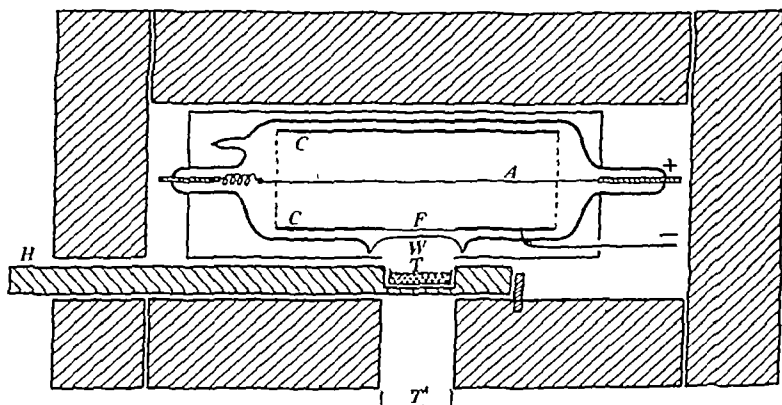


Fig. 1. Tube counter. A, Anode; C, cathode; F, aluminium window; W, thin glass window; H, holder for capsule; T, T', capsule in alternative positions. Lead screens shown shaded.

For counting random impulses at much higher frequencies than this (above 400) the simple registering system employed would have had to be replaced by a more elaborate one with a smaller time of relaxation. (With this end in view an amplifier feeding into a Cenco impulse counter was actually constructed for use in the later stages of the work.)

From these observations with the standard capsules, a curve could be drawn connecting the number of impulses registered per min. (after subtracting the spontaneous discharge rate) with the activity of the material in the capsule: the activity being expressed as the number of ml. of stock radio-P solution employed in preparing 100 mg. of the standard activated P precipitate in the capsule. These calibration curves were nearly linear up to counting rates of 300 impulses per min.

Similar observations were then made of the discharge rate due to each of the capsules containing the phosphate samples derived from the ash of the various tissues of the experimental animals. In a run lasting several hours, check observations of spontaneous discharge and of response to a suitable standard were interpolated at convenient points to control the constancy of the apparatus. Reference to the calibration curve for the day's run, or direct comparison with a standard capsule showing a similar counting rate, then

was in the skeleton. The teeth, skin, muscles and viscera of Group I likewise contained slightly more P than those of Group II. No significant difference was found in the brain, liver or kidneys.

TABLE 3. Mg. P in tissues and excreta

Rat no.	Group I				Group II			
	1	2	3	4	5	6	7	8
Skeleton	442.00	423.28	372.08	384.64	211.67	248.44	207.24	233.92
Incisors	24.69	23.43	22.25	22.58	18.72	23.13	20.38	20.38
Molars	21.09	18.29	18.29	20.10	18.29	16.68	14.90	16.49
Brain	5.75	6.74	6.54	6.14	6.14	6.14	6.14	5.95
Skin	25.51	22.37	17.39	17.39	17.65	19.19	17.45	16.08
Muscles	149.26	114.92	145.95	129.92	101.52	140.84	106.96	123.46
Liver	21.61	17.87	16.88	15.07	15.76	19.17	18.84	15.07
Viscera	41.24	39.22	34.48	35.69	32.09	42.56	32.79	34.48
Kidneys	2.41	5.01	2.86	3.81	3.01	4.84	3.65	3.49
Urine	44.25	43.86	33.12	43.48	27.97	17.10	13.88	1.83
Faeces	15.69	16.67	8.94	15.53	6.76	4.20	5.26	6.76

The figures for excretion of total P over the 90 hr. between injection and killing of the animals are also given in Table 3. These show a higher excretion of P in the urine and faeces of Group I. An accurate estimate of the food intake was not kept so it is not possible to state from these figures if the rats of Group II utilized their P more efficiently. The ratio between the total P excretion of the two groups is almost exactly the same as that between the P content of the two diets. The relative distribution of P between urine and faeces was the same in both groups.

Radio-P. The percentage retention of radio-P by the eight rats is shown in Table 4. This indicates very clearly the increased retention of

TABLE 4

Rat no.	Group I				Group II			
	1	2	3	4	5	6	7	8
Percentage retention of radio-P								
	76.37	71.60	73.69	72.14	59.15	94.60	92.70	96.83
Percentage distribution of radio-P in body								
Skeleton	45.31	54.15	48.79	49.23	36.82	43.16	36.48	32.98
Incisors	2.20	2.01	2.28	2.07	1.70	1.85	2.16	2.63
Molars	0.85	0.66	0.78	0.76	0.84	0.52	0.64	0.48
Brain	0.36	0.48	0.56	0.51	0.57	0.40	0.61	0.61
Skin	5.16	4.64	4.92	3.79	5.04	4.02	4.74	3.93
Muscles	31.15	20.75	26.68	31.58	36.91	33.51	36.48	38.50
Liver	4.77	4.46	4.32	3.65	5.25	3.96	5.95	4.88
Viscera	9.65	11.69	10.85	7.47	11.52	11.42	11.69	14.85
Kidneys	0.58	1.18	0.82	0.88	1.31	1.09	1.13	1.09
Excretion of radio-P as percentage of intake								
Urine	21.2	24.1	26.7	25.0	17.9	4.8	5.3	0.5
Faeces	2.2	3.8	1.9	2.6	1.9	1.1	1.5	2.7

RESULTS

The rats in Group I appeared normal in all respects. The rats in Group II were not sufficiently old when killed to show any gross abnormality. The growth rates of each group were about the same and the weights at death not widely different. Post-mortem examination showed that the soft tissues and internal organs were quite normal in all rats, although the bones were rather soft and deformed in the rats of Group II.

Chemical findings. As previously shown [Gaunt & Irving, 1940], the calcification in Group II was not as good as that in Group I (Table 2). Though the average weights of the rats in the two groups differed little, the bones of the right hind-leg, and in particular the ash of those bones and of the skeleton, were heavier in the rats on the diet with the higher Ca and P content. In Group I the ash represented 50% of the bone, whereas in Group II the corresponding figure was only 38%.

TABLE 2. The chemical analyses of the calcified tissues of the rats

Rat no.	Sex	Wt. rat g.	Wt. skeleton g.	Wt. bone g.	Ash % bone	Wt. tooth g.	Ash % tooth	Total P in body g.	P in calcified tissues g.	P in soft tissues g.
Group I										
1	M	130	2.184	0.4505	49.33	0.1787	71.47	0.7338	0.4878	0.2460
2	M	130	2.035	0.4013	49.68	0.1707	70.97	0.6712	0.4650	0.2062
3	F	112	1.853	0.3423	51.07	0.1528	72.52	0.6367	0.4126	0.2241
4	F	112	1.856	0.3500	50.91	0.1625	73.14	0.6347	0.4273	0.2074
Means		122.5	1.982	0.3860	50.25	0.1662	72.02	0.6691	0.4482	0.2209
Group II										
5	M	111	1.062	0.2815	34.49	0.1341	70.62	0.4248	0.2487	0.1761
6	M	135	1.244	0.3375	38.20	0.1577	72.29	0.5210	0.2883	0.2327
7	F	108	1.058	0.2494	38.24	0.1500	70.46	0.4285	0.2425	0.1860
8	F	110	1.177	0.2397	42.84	0.1462	71.60	0.4693	0.2708	0.1985
Means		116	1.135	0.2770	38.44	0.1470	71.24	0.4600	0.2626	0.1983

The difference in the state of calcification of the rats on the two diets is also clearly shown by the figures for the total P of the body, and for the distribution of this P between the hard and soft tissues. The rats of Group I contained on an average 0.67 g. P and those of Group II 0.46 g. This difference between the total P in the rats of the two groups was largely due to the difference in the P of the calcified tissues. Of the 0.67 g. P in the bodies of the rats of Group I, the P in the hard tissues represented 0.45 g. and that in the soft tissues 0.22 g. The corresponding figures for Group II were 0.26 and 0.20 g.

The P content in absolute amount of the various fractions of the body is shown in Table 3. The chief difference in P content of the two groups

Within half an hour of administration the percentage uptake is about the same in muscle and the skeleton, but later the relative uptake rises faster in bone than in muscle so that after 10 days the skeleton contains nearly twice as much. In the animals of Group I, even after 90 hr., the skeleton had more than $1\frac{1}{2}$ times the radio-P content of the muscles, while in Group II the distribution between muscle and skeleton was still approximately equal. Hevesy & Rebbe [1938] have shown that radio-P is rapidly built into organic compounds in muscle and Artom *et al.* [1937], reporting experiments in which a single rat was injected with radio-P after being kept on a diet very low in Ca and P, found evidence of the synthesis of some 'low' organic P compounds in muscle. No comparison was, however, made by Artom *et al.* with a control animal on a normal diet. It is possible, in view of Nicolaysen's work, that the inorganic plus phosphagen P was being replaced by radio-P more rapidly in the rats of Group II. The writers are unable at present to pursue this farther and the matter must be left undecided.

Skeleton. With the two diets used, better calcification would be expected in the rats of Group I. The uptake of radio-P was likewise greater in this group, both in percentage distribution and in absolute amount. Based, however, on the relative weights of the skeletons, the rats of Group II took up 59% more radio-P than did those of Group I.

The results published by other workers on the uptake of radio-P in conditions of Ca and P deficiency, such as rickets, are somewhat conflicting. Chievitz & Hevesy [1937] and Dols *et al.* [1937] did not find any outstanding difference in the distribution of radio-P in rachitic and normal rats. Dols *et al.* [1938b] found a higher content of radio-P in the bones of rachitic chickens than in those of normal birds. The results reported in the present paper support the findings of the latter workers since per unit of weight, the badly calcified bones took up more radio-P than the normal ones. This would indeed be expected to occur after P depletion.

Teeth. As has been shown in a previous paper [Gaunt & Irving, 1940] and as again illustrated in Table 1. the diet poorer in Ca and P, which had a marked effect upon bone ash, was without influence upon the ash content of the teeth. The metabolism of teeth has been studied by a number of workers using radio-P. Even fully erupted teeth can take up radio-P [Volker & Sognaes, 1940; Hevesy & Armstrong, 1940]. Human teeth take up radio-P at a rate indicating that 1% of the tooth P is replaced every 250 days [Hevesy, Holst & Krogh, 1937]. But the greatest uptake

radio-P by the rats of Group II. The same is seen in the figures of the radio-P content of the urine and faeces, the rats of Group II excreting far less of the isotope than those of Group I.

The percentage distribution of radio-P in the eight rats is also shown in Table 4. The skeleton of the rats of Group I had a much larger relative uptake of radio-P than had that of Group II, but, weight for weight, the bones of Group II took up more radio-P. The muscles of the rats of Group II showed a considerably increased uptake of radio-P, both relatively and also in absolute amount, compared with those of Group I. The viscera of Group II had also a smaller but definitely increased uptake of radio-P. No significant difference in percentage distribution was noted between the teeth, brain, skin, liver and kidneys of the rats of the two groups. Per unit of weight, the teeth of the rats of Group II took up slightly more radio-P than those of Group I.

DISCUSSION

Excretion. Although the difference in the excretion of total P in the two groups over the 90 hr. experimental period appeared to be related solely to the P content of the two diets, there is no doubt from the urinary radio-P figures that the animals of Group II utilized their radio-P much more efficiently. This better utilization of P during P depletion is already well known from the work of Fairbanks & Mitchell [1936], Rottensten [1938] and Owen, Irving & Lyall [1940]. The faecal excretion of radio-P was, on an average, higher in the rats of Group I. This was probably due to a higher radio-P content of the intestinal secretions in this Group.

Muscle. The figures for the distribution of radio-P in the various tissues show that the bulk of the extra radio-P retained by the rats of Group II was deposited in the muscles. No work appears to have been done on the effects of mild P deprivation upon muscle P compounds. Many conflicting reports have, however, been made on the effects of rickets upon the distribution of P in muscle. In one of the most recent of these, Nicolaysen [1936] stated that there was a slight reduction in inorganic plus phosphagen P in muscle during rickets, but no alteration in the other fractions. The results reported above show that there was a slight reduction in the total muscle P of the Group II animals and this reduction was probably in the fraction mentioned by Nicolaysen.

The very large retention of radio-P by the muscles of the rats of Group II is difficult to explain. In normal rats the rate of uptake of radio-P in muscle compared with bone varies with time [Hevesy, 1939].

animals, indicating that rachitic animals either synthesise more lipin or break down less than normal animals.

The slightly higher content of total P in the viscera of the Group I animals was probably due to the higher P content of unpassed faeces.

Other tissues. The total P of the skin was higher in Group I than in Group II. This is probably related to the slightly higher total P content of the soft tissues of the Group I animals. The fact that the radio-P distribution in the skin of the two groups was approximately equal shows that absorption of radio-P after injection was the same in all rats. No difference in total P content or radio-P distribution was found in the brain, liver or kidneys of the two groups. The bulk of the P in the liver would, from consideration of previous work [Artom *et al.* 1937; Perlman *et al.* 1937], be present as lipoid P.

SUMMARY

1. A group of four rats was fed from weaning on a diet containing 0.30% of each of Ca and P, and a further group of four rats was similarly fed on a diet containing 0.12% of each of these elements. When the animals were 49 days old they were injected subcutaneously with a solution containing radio-P and allowed to live for another 90 hr., being continued on the same diets. The growth rate and weights at death were approximately the same in both groups. After being killed, the bodies were divided into fractions comprising the most important tissues and the radio-P content was determined with a Geiger counter.

2. The animals on the diet low in Ca and P contained much less total P than those on the diet higher in Ca and P. The reduction in P in the animals on the poorer diet was almost entirely in the skeleton.

3. The animals on the diet low in Ca and P retained on an average 91.1% of the injected radio-P, those on the diet high in Ca and P retaining 73.3%.

4. The bulk of the extra radio-P retained by the animals on the poorer diet was found in the muscles. The viscera of these animals also retained slightly more radio-P.

5. The skeleton of the rats on the better diet retained a higher percentage of radio-P than that of the rats on the poorer diet. The teeth retained approximately the same percentage in both groups. Per unit weight, however, the bones of the animals on the poorer diet retained 59% more and the teeth 27% more radio-P than those of the other group of rats.

is in growing teeth such as the rat incisor [Chievitz & Hevesy, 1937; LeFevre & Bale, 1939]. The radio-P content is highest at the proximal end of the incisor and lowest at the tip of the tooth [Hevesy *et al.* 1937; Manley & Bale, 1939]. In comparison with the incisor, the rat's molar teeth have a much lower rate of exchange.

As will be seen from Table 4, the percentage distribution of radio-P in the incisors was the same in both groups and the same applied to the molars which took up about one-third of the radio-P content of the incisors. Calculated per unit of weight, however, the incisors of Group II took up 27% more radio-P than did those of Group I. The bones of the rats of this group took up 59% more radio-P than those of Group I. This shows in a further way that the teeth were much less affected by the dietary P deficiency than were the bones. The determination of radio-P is, however, a much more sensitive index of changes in P metabolism than is the estimation of ash content, and the radio-P figures indicate that the teeth had suffered to some extent from the dietary deficiency, though not to the same degree as the skeleton. The estimation of radio-P is probably as sensitive a guide to metabolic changes in the teeth as is histological examination, since Gaunt & Irving [1940] found marked histological changes in the teeth of rats subsisting on the same diet as that which Group II had, though the dental ash values were only slightly altered.

Chievitz & Hevesy [1937] found that after 5 days the radio-P content per g. tissue was higher in the rat's incisor teeth than in the skeleton. A calculation from the figures quoted in the present paper showed that after 90 hr. the radio-P content of the teeth per g. was about half that of the skeleton in Group I and about two-fifths of that of the skeleton in Group II.

Viscera. The high radio-P content of the viscera in both groups was undoubtedly due to the rapid rate of formation of lipoid P in the intestines. Many workers have shown, using radio-P, that the intestines are an important site of formation of phospholipins and that radio-P is incorporated into their structure whether it is ingested or injected [Artom *et al.* 1937; Fries, Ruben, Perlman & Chaikoff, 1938]. Even 100 hr. after administration, the gastro-intestinal tract still contains a large amount of radio-P as lipin [Perlman, Ruben & Chaikoff, 1937]. The viscera of the rats of Group II contained slightly more radio-P than those of the rats of Group I. This may be correlated with the observation of Dols, Jansen, Sizoo & Barendregt [1938a] that the lipins of rachitic animals contain more radio-P after administration of this isotope than those of normal

GLYCOGEN AND ADIPOSE TISSUE

BY E. TUERKISCHER AND E. WERTHEIMER

*From the Laboratory of Applied Physiology, Hebrew University,
Jerusalem**(Received 14 August 1941)*

It has been shown by Hoffmann & Wertheimer [1927] and by Wertheimer [1928] that rats or dogs which are placed after fasting on a diet rich in carbohydrates transiently store a polysaccharide in their adipose tissue. The polysaccharide is precipitable by the method of Pflüger and is presumably glycogen. Recent investigations [Loew & Kréma, 1929; Scoz, 1932*a*; Schoenen, 1932; Wetzel & Held, 1936; Hausberger & Gujot, 1937; Hausberger & Neuenschwander, 1939] have confirmed and added to this finding.

The experiments described in the present paper complete and extend the earlier investigations and deal more particularly with (1) the identification of the polysaccharide deposited in adipose tissue as glycogen, (2) the particular factors which induce the storage of glycogen in fatty tissue, and (3) the ultimate fate of adipose tissue glycogen *in vivo*.

METHODS

Experiments on an extensive scale could only be carried out with rats. These were of the laboratory breed and were fed until the beginning of the experiment on the ordinary oat, bran and vegetable diet. Dogs deposit relatively greater quantities of glycogen in adipose tissue, but are comparatively difficult to maintain under standardized conditions. Pflüger's micro-method for the determination of glycogen was found to be applicable to adipose-tissue glycogen. Sugar was determined after hydrolysis according to the method of Folin-Wu. The results are presented as g. glucose/100 g. fresh tissue. Total carbohydrate content was determined in fatty tissue by the method of West, Scharles & Petersen [1929]. Phosphate was determined by the method of Lohmann & Jendrassik [1928].

6. No important difference between the two groups was noted in the other tissues.

7. The technique of estimation of radio-P is described in detail.

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The experiments described in the present paper complete and extend the earlier investigations and deal more particularly with (1) the identification of the polysaccharide deposited in adipose tissue as glycogen, (2) the particular factors which induce the storage of glycogen in fatty tissue, and (3) the ultimate fate of adipose tissue glycogen *in vivo*.

METHODS

Experiments on an extensive scale could only be carried out with rats. These were of the laboratory breed and were fed until the beginning of the experiment on the ordinary oat, bran and vegetable diet. Dogs deposit relatively greater quantities of glycogen in adipose tissue, but are comparatively difficult to maintain under standardized conditions. Pflüger's micro-method for the determination of glycogen was found to be applicable to adipose-tissue glycogen. Sugar was determined after hydrolysis according to the method of Folin-Wu. The results are presented as g. glucose/100 g. fresh tissue. Total carbohydrate content was determined in fatty tissue by the method of West, Scharles & Petersen [1929]. Phosphate was determined by the method of Lohmann & Jendrassik [1928].

For purposes of comparison, series of experiments were necessary to define the conditions under which glycogen storage in adipose tissue was maximal. It was found that in general young animals gave the best results. Prolonged, partial starvation was more effective than a brief but complete fast. Fasted, sexually mature females may contain much more fat than males. Standard experiments were therefore conducted as follows: young male rats weighing 95–120 g. were maintained for 7–10 days on a diet which induced a loss of body weight of about 20%. The rats lost about 2–3 g. daily. Greater weight losses were undesirable. For some time subsequently the rats were allowed to consume as much food as they desired of the following composition: carbohydrate, 70% (45% starch and 25% cane sugar); protein (casein), 20%, fat, 10%; mineral salt mixture, 4 g.; dry yeast, 2 g.; cod-liver oil, 25 drops. Glycogen was estimated in groin, in testicle and perirenal adipose tissue.

All three types of fatty tissue were quantitatively removed and carefully mixed on an ice-cooled plate. Weight was determined by a hand balance. In a few cases separate analyses of each of the stated types was undertaken, but showed no definite and reproducible differences between them. Interscapular fat, the so-called brown, fat body, was separately investigated.

RESULTS

Identification of the polysaccharide in adipose tissue

The adipose-tissue polysaccharide could be readily and completely hydrolysed to a fermentable monosaccharide by $N/1$ H_2SO_4 . The iodine stain of the purified polysaccharide was identical with that given by similarly purified liver glycogen. A sample of a material purified by Kerr's method [1938] gave the following analysis: Optical rotation determined in 0.87% solution in $\frac{1}{2}$ dem. tube: $[\alpha]_D^{20} + 201$; on hydrolysis 0.0213 g. gave 0.020 g. sugar; nitrogen and P_2O_5 absent. A sample purified according to Somogyi's method [1934] gave the following values: $[\alpha]_D^{20} + 196$; 0.0696 g. on hydrolysis with $D/1$ H_2SO_4 gave 0.0697 g. glucose by the method of Somogyi. In view of these data the polysaccharide deposited in adipose tissue of fasted animals placed on a carbohydrate-rich recovery diet is glycogen.

To determine whether glycogen in adipose tissue was easily soluble (lyo-glycogen), or was closely bound to the tissue (desmo-glycogen), the method of Willstätter & Rohdewald [1934] was used. The following results are typical: a dog was maintained for a week on a starvation diet and was then given a carbohydrate-rich ration during 2 days. Analysis

gave: total fat-tissue glycogen, 1.06%; desmoglycogen, 0.615%; lyoglycogen, 0.32%. It is therefore clear that the greater part of the glycogen in fat tissue is in a closely bound form.

Glucose was only demonstrable in the fatty tissue in traces, if at all. Its presence was questionable, even after incubation under conditions which favour glucose formation. In a series of experiments parallel determinations were made of glycogen and of total carbohydrate by the method of West *et al.* [1929]. Typical data are presented in Table 1.

TABLE 1. Glycogen and total carbohydrate content of adipose tissue

Glycogen %	Total carbohydrate %	Remarks
0.76	0.85	Tissue left for 3-5 min. on ice before analysis
0.96	1.06	
0.17	0.21	
0.32	0.35	
0.35	0.41	
0.19	0.26	Tissue immersed in hot KOH solution for glycogen determination immediately after death
0.59	0.61	
0.42	0.42	
0.60	0.66	

The difference between the glycogen and total carbohydrate level is very small only if the analysis is carried out immediately after death. It may therefore be assumed that much and possibly all the carbohydrate, other than glycogen, is formed after removal of the adipose tissue from the parent organism.

Storage of adipose tissue on ice for 15 min. caused a 12-20% decrease in glycogen content and a corresponding increase in the quantity of unknown carbohydrate. The nature of the latter product will be considered separately. In any case, the greater part of the carbohydrate material deposited in fat tissue during recovery feeding is clearly glycogen.

Occurrence of glycogen in adipose tissue

Scoz [1932*b*] claims that glycogen and glucose are normal constituents of fatty tissue. He emphasizes in particular that glycogen in this tissue is also increased when the metabolism of the organism is changed over from the state of equilibrium to one of hunger and mentions the possibility that adipose tissue fat may be convertible into carbohydrate. Adler-Mönnich & Tiberi [1937] claim furthermore that sugar formation from fat can be obtained with adipose tissue *in vitro*. Experiments concerning these questions are dealt with in Table 2.

The term 'traces' is used in the table to indicate a glycogen content of less than 0.03%. The experiments showed that when rats are either fasted, maintained continuously on a carbohydrate-rich diet, transferred

TABLE 2. The influence of diet on the glycogen and total carbohydrate content of adipose tissue

Series	No. of exps.	Diet	Glycogen	Total carbohydrate
1	10	Continuous basic carbohydrate ration 8 days	Traces	Traces
2	6	As in (1), then fasted for 3 days	"	"
3	6	As in (1), then maintained on a starvation diet until loss of body weight was 20 %	"	"
4	6	Thyroxine continuously administered to a total dose of 4 mg. in 10 days. Phloridzin-hunger. Dinitrophenol in a dose of 30 mg. per 100 g. body weight	"	"
5	5	Continuously maintained on a ration composed of 70 % casein, 20 % carbohydrate and 10 % fat or meat	"	"
6	5	Maintained for 6 days on a ration as in (5), then 2 days on ration as in (1)	"	"

from such a diet to a starvation ration, or changed from a protein-rich to carbohydrate-rich diet, neither glycogen, nor any other polysaccharide is formed in the adipose tissue in appreciable and definitely demonstrable quantities. Even when intensive formation of carbohydrate is necessary for the organism, e.g. as in thyroxine, phloridzin, or dinitrophenol poisoning, no increase of carbohydrate in the fatty tissue was demonstrable.

It may be stated in the light of all the experiments now available that glycogen deposition only occurs in the adipose tissue of rats which show an increase in body weight, when placed on their normal diet after a prolonged fast.

Time relationship between recovery feeding and deposition of glycogen in adipose tissue

Under standard conditions, as described above (loss of 20 % body weight in fast followed by recovery on 70 % carbohydrate diet), glycogen deposition in adipose tissue takes the time course shown in Table 3. These results suggest that the glycogen formed during recovery feeding after starvation has a specific and limited role to play, and that this role is limited to a restricted time, after which glycogen is removed from the adipose tissue through the establishment of a new state of equilibrium.

The loss in weight which is necessary for the deposition of adipose-tissue glycogen need not be brought about simply by hunger. Glycogen deposition will also occur on addition of 8 % dry yeast to the ration, after a 20 % loss in weight has been brought about by absence of vitamin B

TABLE 3. Glycogen percentage in adipose tissue and liver after recovery diet

Duration of recovery diet	...	0	1-2 hr.	5 hr.	1 day	2 days	3 days	4 days	5 days	6-8 days
No. of exps.	...	8	8	7	28	10	7	7	8	8
Glycogen in adipose tissue	Tr.	0.05	0.24	0.60	1.05	0.48	0.03	0.18	Tr.	2.8
Liver glycogen	—	3.0	5.2	6.8	3.2	3.5	3.7	2.7	—	—
Glycogen percentage in interscapular fat										
Duration	...	0	1-2 hr.	5 hr.	1 day	2 days	3 days	4 days	5 days	6-8 days
No. of exps.	...	6	4	11	26	15	6	5	—	8
Glycogen in interscapular fat	Tr.	0.37	1.02	1.48	0.90	0.56	0.22	—	Tr.	—
Glycogen percentage in adipose tissue after vitamin B-free diet										
Duration	0	5 hr.	1 day	2 days	3 days	4 days	5 days	5 days
No. of exps.	8	2	4	3	5	3	2	2
Glycogen in adipose tissue	Tr.	Tr.	Tr.	Tr.	0.2	0.45	0.54	0.19	Tr.	Tr.
Liver glycogen	0.82	—	—	4.4	4.0	2.35	—	—

complex (Table 3). Glycogen deposition in this case is delayed. Possibly during the first day after yeast is added a mechanism, which was paralysed by the absence of vitamin B, may be set in motion within the adipose tissue [McHenry & Gavin, 1938].

The influence of fat-soluble vitamins A and D on glycogen and fat deposition in adipose tissue was also studied. Young rats of 80-100 g. were maintained on a vitamin A and D-free ration until growth disturbances were definitely evident. The animals were then given a vitamin A and D-free starvation diet, and so maintained until they had lost about 15% of their weight. They were then transferred to a carbohydrate-rich vitamin A and D-free ration. In five experiments it was uniformly observed that a normal and adequate deposition of glycogen and fat in adipose tissue had occurred. The amount of glycogen deposited after 6 hr. was 0.7% in the adipose tissue and 2.2% in the interscapular fat.

Deposition of glycogen after starvation is far more marked and occurs more rapidly in the brown fat body than in other fatty tissue (Table 3). In this respect it is comparable to liver, with the difference, however, that brown fat tissue is already empty of glycogen within 4-5 days.

It was noted that the glycogen levels attained in adipose tissue are subject to seasonal fluctuations. In winter lower levels were noted than in summer (June to November), and deposition of glycogen only began 5 hr. after feeding, though otherwise the time course of deposition was unchanged. At the commencement of summer the glycogen values of adipose tissue were particularly high. In ten experiments in May the

average glycogen deposition after 1 day recovery feeding was 1.18% adipose tissue, and 4.2% for interscapular fat, most of the value the latter being between 4 and 6%.

Influence of diet on glycogen deposition in adipose tissue

Experiments with different diets are set out in Table 4. On a dominantly protein ration given after fasting, glycogen deposition in adipose tissue does not occur. Three separate high-protein rations

TABLE 4. Influence of diet on glycogen deposition in adipose tissue

Diet	No. of exps.		Adipose tissue glycogen %		Liver glycogen %		Remarks
	1 day	2 days	1 day	2 days	1 day	2 days	
70% carbohydrate } 20% casein } 20% fat }	10	6	0.58	1.00	6.8	3.2	
40% carbohydrate } 40% casein } 20% fat }	5	5	0.20	0.17	3.3	1.2	
40% carbohydrate } 40% fat } 20% casein }	5	5	0.09	0.10	1.2	1.65	
70% casein } 20% carbohydrate } 10% fat }	4	7	0.02	0.05	2.85	1.9	After 3, 4, 8 d traces of tissue glycogen
85% casein } 15% carbohydrate }	4	4	Tr.	0.034	—	—	
Meat	2	2	Tr.	Tr.	—	—	
85% carbohydrate } 15% fat }	5	5	0.30	0.215	7.9	4.1	
1.75-3.2 g. glucose by stomach tube	6 (6-8 hr.)		Tr.		2.6		

this result: (1) 70% casein, 20% carbohydrate and 10% fat; (2) 80% casein, 10% carbohydrate and 5% fat; (3) meat only. It was further found that on a ration containing protein and carbohydrate in equal proportions (45% protein, 45% carbohydrate and 10% fat), glycogen deposition in adipose tissue is much reduced. The same result was obtained with a ration containing carbohydrate and fat in equal proportions (40% fat, 40% carbohydrate and 20% casein). However, in this latter case it was probable that the deposition of glycogen was being inhibited by the deposition in the adipose tissue of fat from the ration itself. With a ration rich in carbohydrate, poor in fat and lacking in protein (85% carbohydrates, 15% fat), glycogen deposition in the adipose tissue was considerably less than on the standard diet.

Large quantities of glucose (1.75-3.2 g.) administered by stomach tube after fasting, though absorbed rapidly, induced no deposition

glycogen in ordinary fatty tissue within 6–8 hr. and only a slight glycogen deposition (0.5%) in the interscapular fatty tissue. Corresponding control experiments using 0.9% NaCl solution instead of glucose did not influence deposition of glycogen in adipose tissue.

In vitro experiments

An attempt was made to obtain a deposition of glycogen in adipose tissue from fasted animals *in vitro*. Various conditions were tried. Unless otherwise specified, the experiments were carried out in a water bath at 37° C. under continuous passage of oxygen. The suspension medium was phosphate-Ringer, as indicated by Krebs. In parallel experiments tests for the appearance of glucose in the suspension medium were carried out. Synthesis of glycogen or production of sugar could not, however, be demonstrated *in vitro*. A finely minced suspension of 0.4 g. adipose tissue from a fasted rat in 2 c.c. phosphate-Ringer solution was used for each test in the following series:

(1) Adipose tissue and 10 mg. glucose, incubated for $\frac{1}{2}$ and for 4 hr. with or without hexokinase (as in experiments on synthesis of muscle glycogen by Willstätter & Rohdewald [1940]) and with or without addition of either monoiodoacetate 1:1500, or KCN 1:2500 and 1:5000.

(2) Adipose tissue and either $M/50$ pyruvate, lactate, or butyrate, incubated for 4 hr.

(3) Adipose tissue and 10 mg. Cori ester, incubated for 15 min., 2 hr. or 20 hr., with, or without addition of $M/200$ NaF (as in the experiments of Ostern, Herbert & Holmes [1939]).

An attempt was then made to obtain deposition of glycogen in fatty tissue by perfusion experiments, in which 0.5% glucose-phosphate-Ringer solution was added to blood in the proportion of 2:1. In cats perfusion was effected via the aorta and in rats via the cava. The perfusion fluid was saturated with oxygen. Glycogen deposition could not, however, be demonstrated.

Deposition of fat in adipose tissue during recovery feeding on a carbohydrate-rich diet after fasting

Rats, maintained under the standard conditions already described, were fasted, and after various intervals of recovery feeding, killed and examined. The groin, testicle and perirenal adipose tissue was carefully collected and weighed. The results are summarized in Table 5.

These results, together with those in deposition of glycogen, indicate that a correlation probably exists between the deposition of fat and

TABLE 5. Deposition of fat in adipose tissue. Results expressed as g./100 g. body wt.

No. of exps.	Nutrition	Wt. of fat g.
34	Fast	1.02
32	Standard ration: 1 day	1.33
23	2 days	1.65
7	3 days	1.86
14	4 days	2.52
19	5-10 days	2.75
7	without fast	2.90
7	70% casein 2 days	0.94
6	70% casein 4 days	1.50
6	70% casein 9 days	1.88
9	40% casein 2 days	1.25
10	40% fat 6-10 days	2.70

glycogen in adipose tissue. Deposition of fat is definitely demonstrable 1 day after recovery feeding, continues on subsequent days side by side with glycogen deposition, attains a maximum on the 4th day, when the main deposition of glycogen is already at an end and is almost negligible in subsequent periods when glycogen deposition is both small and uncertain. The disappearance of adipose glycogen may thus be said to mark the re-establishment of a fat equilibrium in the organism. It is remarkable, indeed, that within 4 days, and after a considerable loss of body weight, the fat depots of rats on a standard diet should be fully replenished and that, moreover, the completion of this process should coincide with that at which deposition of glycogen in the adipose tissue ceases. When rats were maintained on a protein-rich ration, the course and character of fat deposition were markedly different. Fat deposition was delayed and the amount was smaller than under standard conditions. With the high fat ration the amount of adipose fat deposited after 6-10 days equalled that found on the standard ration. In this case deposition in adipose tissue of fat from the ration itself may be assumed to have taken place.

Glycogen and fat deposition in the adipose tissues of herbivora

The course of deposition in guinea-pigs and rabbits is very different from that found in rats, dogs, and cats (unpublished experiments). Experiments with the herbivores followed the exact procedure described above for rats. The results are given in Table 6. The low total values, irregular nature and considerable extension of the period of glycogen deposition in guinea-pigs are striking. The maximum values were attained on the average after 5 days; zero value was only attained after 2 weeks of recovery diet. The comparatively uniform liver and muscle glycogen values throughout the period of recovery feeding averaged 7.2 and 0.84 %

TABLE 6. Glycogen deposition in the adipose tissue of guinea-pigs and rabbits

(a) Guinea-pigs.

Recovery diet days	Gain in body wt. g.	Glycogen %			Exps. with negative glycogen deposition	Exps. with significant increase of fat in adipose tissue	No. of exps.
		Groin fat	Abdominal fat	Inter-scapular fat			
1	36	0.12	—	0.16	1	—	5
2	40	0.10	0.10	0.22	1	—	5
3	52	0.23	0.08	0.07	1	—	4
4	54	0.10	0.11	0.07	2	—	5
5	56	0.30	0.37	0.30	—	1	5
6	70	0.15	0.23	0.14	—	3	5
7-8	79	0.16	0.15	0.24	—	5	5
9-11	89	0.18	—	0.15	—	4	4
12-13	97	0.11	—	0.08	2	5	5
14	110	Tr.	—	Tr.	3	3	3

(b) Rabbits.

1	168	0.16	—	0.23	0	—	5
2	172	Tr.	—	0.13	3	—	5
3	184	0.05	—	0.052	2	—	4
4	184	0.05	—	0.03	2	—	4
5	247	0.042	—	0.06	2	—	4
6-10	266	Tr.	—	Tr.	6	—	6

respectively. Fat deposition in guinea-pigs is much less regular and in the first days so small as to be insignificant. Appreciable fat increments in adipose tissue were only recorded after a week of recovery feeding.

In rabbits glycogen deposition is smaller and even less regular. Glycogen deposition was uniformly recorded here only in the first day of recovery feeding. Fat deposition was so uncertain as to render any quantitative expression of the results impossible.

Stimulation of adipose tissue

It has been shown already [Wertheimer, 1926, 1927] that scission of suitable nervous connexions renders popliteal fat relatively immune to exploitation during phloridzin starvation. Hausberger [1935] was able to cut the nerve supply of the interscapular fat body of the mouse on one side only, and showed that an increase of glycogen and in its wake an accumulation of fat already became noticeable in the denervated half of the interscapular fat body 10 hr. after denervation. The fat content of the denervated side exceeded that of the control side also during starvation. Beznák & Hasch [1937] have made similar observations.

In view of the technical difficulty of isolating and stimulating the nerve supply of the fat body, experiments on the influence of a direct, purely mechanical type of stimulus, viz. massage, seemed desirable. Groin fat tissue was chosen for these experiments. One side of the groin was massaged, while the second served as a control. Each massage

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2	40	0.10	0.10	0.22	1	—	5
3	52	0.23	0.08	0.07	1	—	4
4	54	0.10	0.11	0.07	2	—	5
5	56	0.30	0.37	0.30	—	1	5
6	70	0.15	0.23	0.14	—	3	5
7-8	79	0.16	0.15	0.24	—	5	5
9-11	89	0.18	—	0.15	—	4	4
12-13	97	0.11	—	0.03	2	5	5
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period lasted 2 min. and this was repeated at equal time intervals four times daily. The massage was of the usual type.

The first question to which an answer was sought was whether massage could influence deposition of glycogen on the adipose tissue. Normally, approximately equal quantities of adipose glycogen are found on opposite sides of the groin. At the very most a difference of 10% may occur. The usual experimental procedure was employed. Fasting was instituted to obtain a loss of 20% body weight and was followed by a period of recovery feeding during which massage was performed on one side of the groin only. The animals were then killed and the groin fat and groin muscle tissue of both sides isolated (over ice). The results of the experiments are presented in Table 7, and show that muscle glycogen remains the same on both sides, whereas two massage treatments only—applied 6 hr. before death—suffice to lower the adipose glycogen level on the massaged side.

TABLE 7. Effect of massage on adipose-tissue glycogen during recovery diet

Body wt. g.	Groin-fat glycogen %		Glycogen in abdominal fat %	Glycogen in muscle %		Recovery diet days	Mas-sage, side	Duration days	Rest, days after mas-sage
	Left	Right		Left	Right				
101	0.19	0.60	—	0.61	0.60	1	L.	1	—
105	0.27	0.68	—	0.67	0.77	1	L.	1	—
84	Tr.	0.165	0.134	0.39	0.44	1	L.	1	—
106	0.03	0.116	0.133	—	—	2	L.	1	—
98	0.44	0.65	0.98	0.44	0.44	1	L.	Only twice	—
261	Tr.	0.126	0.20	0.31	0.32	2	L.		—
115	0.08	0.28	0.23	0.37	0.44	2	L.	2	—
105	0.214	0	0.266	0.38	0.38	1	R.	1	—
250	0.09	Tr.	0.15	0.40	0.45	1	R.	1	—
103	0.54	0.22	0.61	0.40	0.42	2	R.	2	—
107	0.24	0.32	0.47	0.80	0.80	3.5	L.	2	1.5
145	0.29	0.25	0.22	0.54	0.50	4.5	L.	2	2.5
210	0.167	0.157	0.22	0.50	0.50	1	—	1 electrical stimulation	
95	0.42	0.43	0.53	0.44	0.50	1	—	1 electrical stimulation	
117	0.255	0.244	—	—	—	1	—	1 direct electrical stimulation of fat tissue	

One-sided electrical stimulation by induction shock applied along the full length of the adipose tissue at repeated intervals failed to affect the glycogen level.

More prolonged treatment by massage was next tried to see if the deposition of fat was affected. The same experimental procedure was followed, except that the massage periods were spread over 3–8 days, since fat deposition only attains maximum level several days after the beginning of recovery feeding. Where massage was not applied, the adipose

TABLE 8. Effect of massage on fat deposition under various conditions

a) During a normal recovery diet.

Body wt. g.	Extracted fat		Difference %	Duration of massage days	Side
	Left g.	Right g.			
110	0.911	0.763	16	6	R.
116	0.941	0.699	26	8	R.
109	0.755	0.781	3	5	L.
105	0.490	0.628	22	4	L.
102	0.384	0.434	11.5	4	L.
100	0.338	0.449	24	3	L.
120	0.507	0.643	21	6	L.

b) During continuous maintenance on a normal diet.

121	0.372	0.383	3	5	L.
110	0.672	0.724	7	5	L.
112	0.614	0.561	8.6	7	R.
115	0.498	0.603	16.6	8	L.
130	0.760	0.899	18	9	L.
120	1.037	0.808	22	8	R.

c) During a fat-rich recovery diet.

128	0.694	0.792	12	6	L.
109	0.710	0.811	12	6	L.
110	0.998	0.745	15	7	R.
93	0.533	0.610	12.6	4	L.
100	0.487	0.585	16.7	4	L.

fat content of both groin sides was practically equal. The results of some typical experiments are presented in Table 8.

The figures show that the fat content of the massaged groin is uniformly smaller than that of the unmassaged. The difference in fat content is already noticeable 3 days after beginning the application of massage; the effect of the duration of massage on the fat content is not clear from these experiments however, as individual differences between the animals were too considerable for any decision in this respect. The fat levels of the massaged and unmassaged groin show differences of 11-37% (eighteen experiments) and a mean difference of 20.7% (in one experiment a difference of only 3% was found). Massage carried out during the period of weight loss (starvation, or starvation plus phloridzin) failed to effect a considerable change in the rate at which the adipose tissue was depleted.

When the groin of rats which have not been starved and which are maintained on a normal diet is massaged (twelve experiments), the fat content of the massaged groin falls below that of the unmassaged side, but does so rather slowly (Table 8). After 7 days' massage the lesser fat content of the massaged groin is clearly discernible. The maximum difference between massaged and unmassaged groin was found after 8-10 days when the average decrease was 23%.

If in recovery experiments as described above, the usual carbohydrate-rich diet is replaced by a fat-rich but carbohydrate-poor diet (40% fat, 40% carbohydrate, 20% protein), and massage is applied during recovery feeding lasting 4-7 days, the differences noted are smaller than with a carbohydrate-rich diet—13.6% on the average, as against 20.7% in the carbohydrate diet experiments (Table 8). A similar relationship was established in experiments on the effect of prolonged massage treatment (8-10 days) which was not preceded by starvation. On the fat-rich diet the difference produced by prolonged massage was found to be 11%, as against 23% on a carbohydrate-rich diet.

Mechanical stimulation by massage thus inhibits both glycogen and fat deposition in adipose tissue under the conditions described. The inhibitory effect on fat deposition is evident even when the rats are not starved but are continuously maintained on a normal diet, though in this case massage must be prolonged to be effective. Since the inhibitory effect of massage is generally found to be smaller on a fat-rich rather than a carbohydrate-rich recovery diet, it seems probable that massage inhibits especially neogenesis of fat from carbohydrate in adipose tissue. The same experiments also demonstrate the extreme reactivity of adipose tissue metabolism to relatively feeble stimuli, e.g. massage applied for 2 min. four times daily. If the effect is mechanical and due to the pressing out of fat from the adipose tissue or to the facilitation of fat absorption by the vascular system, the extension of the effect to the process of adipose tissue depletion in starvation is to be expected. In fact, however, massage was found to be without effect on the latter. Moreover, if the effect of massage was merely mechanical, massage should be at least equally effective in experiments where the normal diet is given throughout, as in recovery experiments.

Influence of toxins on the deposition of glycogen and fat in adipose tissue

Experiments were undertaken to elucidate the effect on adipose glycogen deposition of poisons which are known to affect carbohydrate metabolism. For the first experiments bacterial endotoxins whose influence on carbohydrate metabolism has been the subject of systematic study [Delafield, 1932; Olitzki, Leibowitz & Berman, 1937] were chosen. A dead, washed preparation of *Salmonella typhi murium* was used.¹ The dose was of sufficient strength to cause an appreciable decrease in the glycogen content of the liver and was separately assayed therefore for each preparation.

¹ We are indebted for this preparation to Dr L. Olitzki and to Mr P. Koch.

In the first experiments, the effective dose (1 mg./100 g. body weight) was injected twice, 24 and 4 hr. before the animals were killed. In later experiments only a single injection, 24 hr. before the determination, was given. The following results were obtained:

No. of exps.	Body weight of rats g.	Liver glycogen %	Adipose tissue glycogen %	Inter- scapular fat glycogen %	Blood sugar mg. %	Remark
3	98	1.42	Traces	0.15	89	Two injections of 1 mg. toxin
6	115	4.4	Traces	0.83	120	Single injection of 1 mg. toxin

The bacterial toxin prevented deposition of glycogen in typical adipose tissue, inhibited deposition of glycogen in interscapular fatty tissue and in liver, and failed to influence the glycogen content of muscle. The food intake by the poisoned animals was lower than normal, though not to an extent which might affect the amount of glycogen deposited. Control experiments with normal animals which received similarly restricted quantities of food confirm this view. The effect of the toxins on adipose-tissue, glycogen was further investigated as follows: After a day of recovery feeding, groin fat from one side was removed and analysed. At the same time an effective dose of toxin was injected. The fat of the remaining groin side was sampled in experiment (1) after 1 hr., in experiment (2) after 4 hr. The following glycogen values were recorded: in experiment (1) at the beginning 0.19%, after 1 hr. 0.15%; in experiment (2) at the beginning, 0.27%, after 4 hr., 0.

By similar methods information as to the influence of toxin on fat deposition was obtained. In these experiments the rats received injections of effective toxin in daily doses for 4-5 days during recovery feeding. Unpoisoned rats contained 2.5 g. fat per 100 g. body weight after this time. The toxin-treated rats yielded the following values:

No. of expts.	Adipose tissue, g./100 g. body weight	Liver glycogen %	Muscle glycogen %
7	1.33	1.7	0.40

In other experiments the effect of administration of strychnine on the glycogen content of adipose tissue was examined. The procedure for these tests was as follows: After 1 day of recovery feeding, as in the standard experiments, groin fat was removed from one side under light ether narcosis. The operated animals were then given cramp-inducing doses of strychnine (0.12 mg. strychnine sulphate per 100 g. body weight) and

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killed after $\frac{1}{2}$ -1 hr. This treatment exerted no measurable effect on the adipose tissue glycogen though it caused a marked fall in the glycogen content of muscle. The following results are typical:

No. of exps.	Adipose-tissue glycogen %		Liver glycogen %	Muscle glycogen %
	Before strychnine	After strychnine		
6	0.70	0.72	6.8	0.09

The influence of endocrine glands on the deposition of glycogen and fat in adipose tissue

If deposition of glycogen and the accompanying deposition of fat in adipose tissue are not passive processes of storage but co-ordinated activities of the adipose organ, their subjection to hormonal regulation may be expected.

(a) *Thyroidin*. The experimental animals received a daily portion of 10 mg. thyroidin for 4-5 days as a supplement to their ordinary diet. After this time the food given was so far reduced as to effect a 20% loss in body weight within 6 days. Recovery feeding with a supplementary portion of 10 mg. thyroidin per day followed. In all, the rats were subject to the action of thyroidin over a period of 10-16 days. The effectiveness of the thyroidin treatment was not only evidenced by the decrease in the amount of liver glycogen but was also reflected in the results of gas exchange tests which revealed an increase in the rate of oxygen consumption of 40-50%.

Table 9 and Figs. 1a and 1b show that the time curves of glycogen deposition in normal and thyreotoxic rats are markedly different. The curve in thyreotoxicosis is much steeper, its high peak is attained within the first day which also sees the beginning of the descent. The figures for liver glycogen in thyreotoxicosis are almost negligible. It seems probable that here too a disturbance in deposition accompanies the enhanced rate of utilization. In adipose tissue, glycogen storage is not inhibited but is actually favoured, though concurrently combustion or other means of utilization of glycogen are stimulated.

The enhanced utilization of glycogen in the adipose tissue of thyreotoxic animals may be demonstrated by the following procedure: Fasting until 20% loss of body weight occurs, followed by 24 hr. standard diet, then sampling of groin fat from one side, and after 1 hr. sampling from the second side, both operations being carried out under amytal narcosis. In normal animals the glycogen content after 1 hr. is practically unchanged, but in thyreotoxic animals the glycogen content drops from

TABLE 9. Glycogen and fat deposition in adipose tissue in thyreotoxic and thyroidectomized rats on recovery diet

Duration of recovery diet	No. of expts.	Glycogen in adipose tissue %	Glycogen in interscapular fat %	Wt. of fatty tissue g.	Glycogen in liver %	Glycogen in muscle %
Thyreotoxic rats.						
0	6	0	0	0.63	Tr.	—
1.5 hr.	2	0.08	0.06	—	Tr.	0.36
5 hr.	5	0.89	1.43	—	0.61	0.58
1 day	18	0.78	0.72	1.16	0.34	0.48
2 days	7	0.41	0.40	1.41	0.64	0.64
3 days	4	Tr.	Tr.	—	Tr.	0.42
4 days	4	0.07	0.03	1.30	Tr.	0.62
6 days	4	Tr.	Tr.	1.26	Tr.	0.5
Thyroidectomized rats.						
5 hr.	2	0	—	—	—	—
1 day	3	0.20	0.88	—	5.6	—
2 days	3	0.42	0.40	—	5.2	—
4 days	2	0.05	0.28	—	5.0	—
8 days	3	Tr.	0.10	—	3.4	—

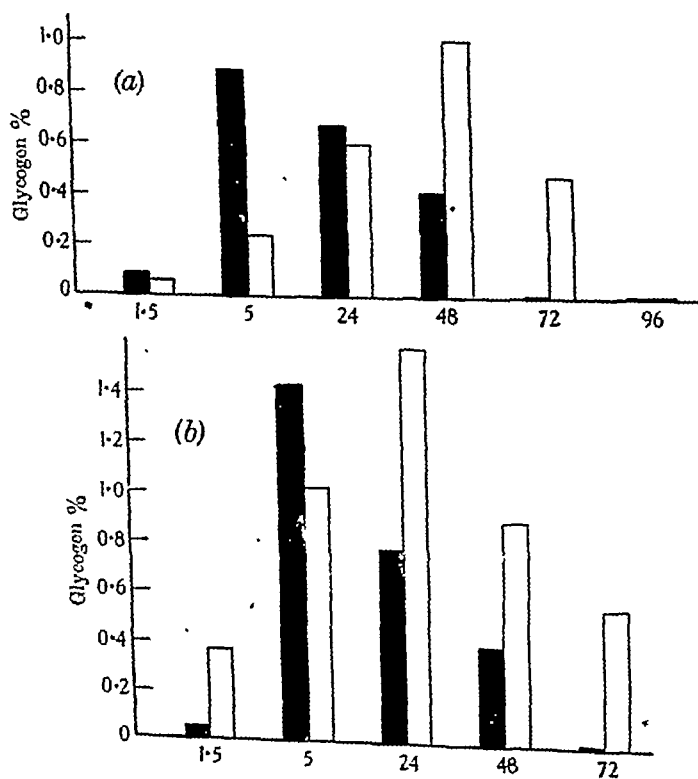


Fig. 1. a, glycogen in adipose tissue; b, glycogen in interscapular fat. Ordinates: glycogen%. Abscissae: duration of recovery diet in hours. ■ Thyroidin-treated animals, □ controls.

0.72 to 0.49 g. %. As may be seen from Table 10, an appreciable drop in the glycogen content also occurs in the other adipose tissues.

1:2:4-Dinitrophenol administered under corresponding conditions exerts a qualitatively similar but quantitatively feeble effect on adipose tissue. Fat and glycogen deposition accompany one another. When glycogen is at peak level an appreciable increase in fat content is evident, but with the disappearance of glycogen the deposition of fat ceases.

(b) *Thyroidectomy*. Thyroidectomized rats deposited less glycogen fat than did normal rats. In no case was obesity observed in these animals (Table 9).

(c) *Insulin*. The method of groin-fat analysis was used. It was found in such experiments that doses of insulin which sufficed to depress the blood-sugar concentration by 50% failed to induce an increase in the concentration of adipose-tissue glycogen (Table 10). In addition, experiments using small doses of insulin which affected the blood-sugar concentration only slightly were carried out but no effect of insulin was found. Also, no effect of insulin on adipose glycogen was observed during fasting.

TABLE 10. Effect of various endocrine substances and dinitrophenol on the glycogen content of adipose tissue

Remarks	Body wt. g.	No. of exps.	Glycogen %						Blood sugar mg. %
			Groin fat		Abdominal fat	Inter- scapular fat	Liver	Muscle	
			Before lb.	After lb.					
Controls	109	6	0.71	0.75	0.70	4.0	6.2	0.72	105
0.03-0.1 mg. adrenaline/100 g.	103	10	0.67	0.65	0.82	3.1	6.6	0.42	240
Insulin	106	5	0.71	0.64	0.81	3.7	4.6	—	50.2
Thyroidin 10 days 10 mg. daily	108	7	0.72	0.49	0.36	0.46	0.68	0.41	126
Dinitrophenol 3 mg./100 g.	109	8	0.82	0.60	0.92	3.3	2.1	0.28	231

(d) *Adrenaline*. Fat was taken from one groin after 1 day of recovery feeding and then adrenaline was injected. Fat was taken from the remaining groin 1 hr. later. Under these conditions no effect of adrenaline on the glycogen content of adipose tissue was discernible. Severe hyperglycaemia was produced. Muscle glycogen was diminished (Table 10).

(e) *Adrenalectomy*. It was observed that adrenalectomized rats which died several weeks after operation and which had not suffered considerable losses of body weight contained only minimal quantities of adipose tissue, such as are only rarely found in normal rats even after most severe weight loss (30-40%). Thyreotoxic rats suffer comparable fat depletion only

exceptionally and after fasting. For the analysis pooled samples of groin, testicle, and perirenal fat of rats weighing 140–200 g. were used. Whereas the adipose tissue in adrenalectomized rats after loss in body weight from 0 to 20% weighed only 0, 10–0, 40 g., the weight of adipose tissue in normal rats under essentially similar conditions was 2–5 g. (eight experiments).

The experiments on the deposition of glycogen and fat in adipose tissue of adrenalectomized animals suggest a possible explanation of the above phenomenon.

Rats weighing 130–170 g. were adrenalectomized by dorsal approach under amytal-ether narcosis. On a diet rich in bread and with Rubin-Kriek salt solution to drink, the operated animals generally recovered rapidly. They were subsequently placed on restricted diet until they had dropped approximately 20% in body weight and were afterwards transferred, as in the preceding experiments, to a carbohydrate-high recovery diet for varying lengths of time. The food intake and consequent weight increase of the adrenalectomized animals was 30–40% below normal. Except as specified the technique of these experiments was the same as that followed previously.

TABLE 11. Glycogen and fat deposition in adipose tissue of male adrenalectomized rats on recovery diet

Duration of recovery diet	No. of exps.	Weight of fat tissue g.	Glycogen %				Blood sugar mg. %
			Adipose tissue	Interscapular fat	Liver	Muscle	
0	6	1.1	0	0	Tr.	0.16	84
5 hr.	3	—	0	0	Tr.	0.19	90
1 day	7	1.0	0.06	0.10	2.1	0.23	100
2 days	7	1.06	Tr.	Tr.	1.96	0.28	96
3 days	4	1.8	Tr.	0.036	0.80	0.23	85
4–5 days	Adren. 3	1.13	0	0.4	1.7	0.28	—
	Controls 3	4.43	0	0.15	2.8	0.44	—
6 days	Adren. 4	1.2	0	0.11	1.5	0.31	91
	Controls 2	5.0	0.11	0.22	4.2	0.63	—

In over forty experiments involving different times of recovery feeding, typical adipose tissue was found to contain at the most only traces of glycogen. Interscapular fat, after extended time intervals, was seen to contain slight quantities of glycogen but considerably less than was contained in the brown fat of control animals after similar time intervals. Deposition of glycogen in liver and muscle is diminished under similar treatment, but remains considerably higher than during hunger. The blood-sugar concentration also remains high (Table 11).

It may be noted that all animals used in the above experiments were in good condition. It was arguable that their inability to deposit glycogen

was conditioned by a lowered food intake and consequent weight loss. Experiments show, however, that this is not the case. Normal rats, and vitamin A- and B-deficient rats showed normal glycogen deposition at subnormal food intake rates. On the other hand, adrenalectomized rats have been known to show normal rise in weight but no accompanying deposition of adipose-tissue glycogen.

When the food intake was restricted during recovery feeding so as to limit weight gain to 6 instead of the normal 14 g. in the first day, the following glycogen values were recorded:

No. of exps.	Adipose-tissue glycogen %	Liver glycogen %	Weight of rats g.
6	0.35	6.8	102

After short recovery times (8 hr.) normal amounts of adipose glycogen were deposited by rats on a restricted recovery diet. Adrenalectomized rats only rarely took as little food or experienced such low rates of gain in weight as were established by restriction of the ration. It appears, therefore, that the adrenal cortex is indispensable to glycogen formation in adipose tissue.

In a recent comprehensive paper, Long, Katzin & Fry [1940] state that glycogen deposition in liver and muscle by adrenalectomized rats is normal if the animals are given daily injections of a suitable dose of NaCl and NaCO₃. In a separate series of experiments, therefore, rats which had lost 20% of their weight were transferred for 2 days to a recovery diet, during which time they also received salt injections as prescribed by Long *et al.* Such rats showed very satisfactory and nearly normal increase in weight. Their general condition was good. In view of the fact that the liver and muscle glycogen content of fasted adrenalectomized rats is generally abnormally low the figures found after recovery feeding may be regarded as evidence of vigorous deposition. The glycogen content of the adipose tissues under the same conditions nevertheless remained near zero level; a value of 0.22% was only found on a single occasion. In interscapular fat a slight amount of glycogen deposition was noted, but this too was abnormally low. Values recorded in these experiments were as follows:

No. of exps.	Weight g.	Weight gain (average) g.	Liver glycogen %	Muscle glycogen %	Adipose- tissue glycogen %	Inter- scapular fat glycogen %	Blood sugar mg. %
5	139	14.5	2.74	0.293	0.051	0.128	125

In fourteen experiments with adrenalectomized rats which were given various quantities (up to 1 mg./day) of desoxycorticosterone acetate (doca organon) for varying time periods, glycogen synthesis in adipose tissue was noted only on two occasions, the levels then obtained being 0.43 and 0.30%. Under similar conditions low amounts or only traces of glycogen occurred in interscapular fat. The averages noted show that apart from a small improvement in the glycogen values of liver, desoxycorticosterone is without effect on glycogen deposition.

Using a fresh extract of cattle adrenals prepared according to Cartland & Kuizenga [1936], it was possible to obtain some deposition of glycogen in adipose tissue in the majority of investigated cases (Table 12). The effect on liver glycogen was more marked. The extract in these experiments was given twice daily in a dosage corresponding to 5 g. adrenal gland for 2-3 days before the determination.

TABLE 12. Effect of adrenal extract on the deposition of glycogen

No. of exps.	Weight g.	Liver glycogen %	Muscle glycogen %	Adipose- tissue glycogen %	Inter- scapular fat glycogen %	Remarks
14	133	3.2	0.32	0.05	0.13	Treatment with desoxycorticosterone
5	140	6.2	0.41	0.31	1.2	Treatment with fresh adrenal extract

It may be noted in connexion with the above observations that semi-adrenalectomized rats behave, as regards glycogen deposition, like normal rats.

Similarly uniform results were also obtained in experiments on the deposition of fat in the adipose tissues of male adrenalectomized rats (Table 11). The occurrence in this case of a slight fat deposition after 3 days' recovery feeding is exceptional. On the fourth and fifth day when fat deposition by normal rats reaches a turning point no deposition at all was observed. The figures for normal animals maintained under similar conditions show the difference most clearly. After extended recovery periods, moreover, an actual loss of adipose-tissue fat by adrenalectomized males was observed. The losses noted after extended recovery times were such as are observed in these animals before death.

Results obtained with adrenalectomized female rats must be considered separately (Table 13). In these animals glycogen was not deposited in the adipose tissues but the deposition of fat in the first days of recovery feeding was frequently considerable though always subnormal. It was

TABLE 13. Glycogen and fat deposition in adipose tissue in female adrenalectomized rats on recovery diet

Adren.	Duration of recovery diet	No. of exps.	Wt. of fat, tissue g. (1, 2-5, 4)	Glycogen %				Blood sugar mg. %
				Adipose tissue	Inter-scapular fat	Liver	Muscle	
	0	6	3.0	0	0	Tr.	0.15	—
Adren.	1 day	3	1.2	0.05	0.14	4.9	0.4	111
Controls		3	1.4	1.18	2.1	7.7	0.46	—
Adren.	2 days	2	2.5	Tr.	0.06	2.35	0.28	108
Controls		2	3.3	0.465	0.233	2.35	0.40	—
Adren.	3 days	1	4.25	Tr.	0.02	1.73	0.29	—
Controls		1	5.23	0.95	2.57	4.70	0.89	—
Adren.	4 days	5	2.7	0	0.11	1.34	0.38	97
Controls		5	3.1	0.16	0.12	3.2	0.48	105
Adren.	6 days	5	1.5	0	0.06	1.35	0.30	102
Controls		5	3.8	0.06	0.06	2.9	0.36	—

found, however, that no new fat formation had occurred here. In medium-sized female rats, as has already been noted, considerable fat reserves (1, 2-5, 4 g.) are retained in the body even after 20% weight loss. The reason for this peculiarity has not been cleared up. The fact itself, however, provides a possible explanation for the findings obtained during the first days of recovery feeding. If the observation time is extended to say 4-6 days the quantity of fat does not continue to rise as in normal rats but rapidly and progressively drops.

It follows from the experiments reported above that the absence of adrenal glands renders fat and glycogen deposition in the adipose tissues impossible.

It should be noted that because of the desirability of greater resistance to the operation animals used for adrenalectomy were of larger size than those normally used in the standard tests. Since the larger animals should have contained more fat than the smaller ones used in the standard test, the use of the larger-sized animals does not therefore vitiate the significance of comparisons with normal-sized rats. In the principal experiments, moreover, some control groups of larger sized rats were examined as may be seen in Tables 12 and 13.

It is further noteworthy that thyroidectomy or castration does not change the effect of subsequent adrenalectomy on the carbohydrate metabolism of adipose tissue.

(f) *Castration.* Castration of either males or females failed to influence glycogen deposition in adipose tissue after various periods of recovery feeding. Obesity of castrated rats was not observed (six experiments).

DISCUSSION

The presence of glycogen in adipose tissue during recovery feeding on ordinary diet after fasting raises questions regarding the manner of its deposition. Since peripheral fatty tissue has been regarded as a tissue of minimal activity, consideration must be given first to the possibility that the deposition of glycogen in this tissue is a passive process. Glycogen must be assumed, on this view, to reach the adipose tissue via the blood stream, though it should be noted that well-preserved adipose-tissue preparations contain glycogen within the cells but not in the intercellular spaces. As glycogen cannot diffuse through the cell membrane it is necessary to conclude that it is formed within the cell. The view that glycogen is formed from tissue fat itself is utterly lacking experimental support. There remains the possibility that during recovery feeding, after fasting, glycogen is synthesized within the adipose cell itself from the sugar with which it is supplied. This latter seems to be the only remaining explanation possible; nevertheless, attempts to demonstrate synthesis of glycogen from sugar or sugar breakdown products after starvation by adipose tissue *in vitro* have so far failed. As against this it should be borne in mind that under corresponding conditions it is also difficult to demonstrate synthesis of glycogen even by liver.

The second question to arise concerns the fate of the glycogen of adipose tissue. A rapid disappearance of this glycogen occurs if the recovery diet is withheld. If recovery feeding on a standard diet is continued the typical time curve of glycogen deposition is obtained: a progressive increase in glycogen values to the end of the second day followed by a decrease which is complete on the fourth day. Two principal lines of explanation present themselves.

(1) Excess carbohydrate is deposited as glycogen in the empty adipose tissue and is later drawn into the main circle of carbohydrate metabolism. (2) Adipose glycogen may be the first step towards the formation of fat from carbohydrates in the adipose tissue cell itself. The latter view is supported by the following: (a) As far as is known all deposition of fat is regularly preceded by deposition of glycogen in adipose tissue. Such has been demonstrated in the deposition of fat during recovery feeding after fasting. A similar precedence has also been observed in the deposition of fat in the embryo [Hausberger & Gujot, 1937], and the same relationship has been shown to hold when the deposition of fat follows denervation of adipose tissue. (b) The parallel course of the time curves of fat and glycogen deposition in rats con-

stitutes strong evidence for the interdependence of these processes. The deposition of fat in adipose tissue of rats approaches a maximum after 4 days' recovery feeding when glycogen disappears from the adipose tissue. (c) Deposition of glycogen in the adipose tissue of guinea-pigs was of another order, smaller in magnitude and prolonged for a period of roughly a fortnight; the period of fat deposition in comparison to that found in rats was also prolonged. (d) Treatments which decrease or prevent deposition of glycogen have been shown to effect that of fat in a similar manner. Such, for instance, is the effect of high-protein feeding. This inhibits deposition of glycogen in the fatty tissue and, if the percentage of protein is sufficient, inhibits the deposition of glycogen completely whilst markedly inhibiting that of fat. Massage diminishes the deposition of both glycogen and fat. A similar influence is exerted by certain bacterial toxins. In thyreotoxicosis and during the first stage of recovery feeding a considerable deposition of both glycogen and fat occurs; glycogen deposition then diminishes very rapidly and at the same time fat deposition comes to an end. The best example of the parallel nature of fat and glycogen deposition is perhaps presented by adrenalectomized rats. These fail to deposit glycogen in adipose tissue during recovery feeding but are also unable to deposit fat. The adipose tissue therefore grows ever leaner until at death only traces of fat can be found, even though the loss of body weight may not be considerable.

It is therefore very probable that rat adipose tissue is able at times to effect the conversion of glycogen into fat. It is not to be inferred therefrom that such conversion is impossible to other tissues, particularly to liver. It is also self-evident that in addition to the form of fat deposition which has here been described other forms exist, of which the longest known is that in which fat is deposited directly from the fat of the diet. On a high-fat diet (40%) for instance, the deposition of glycogen in adipose tissue is very slight but that of fat is abundant. Also on a high-protein diet, fat deposition does not occur via glycogen.

The prevalent opinion as to the nature of adipose tissue ascribes to the latter the purely passive role of a fat-receiving depot. The amount of fat stored in the depot is supposed to be dependent on the nutrient content of the blood. The genesis of fat from carbohydrates is regarded as an exclusive function of liver tissue. The replenishment of the fat depot is supposed to take place by an infiltration of fat molecules through the cell walls as through a sieve [cf. for instance Maximow, 1927]. Rosenfeld, however, as long ago as 1902, expressed the opinion that fat might be formed from carbohydrates within adipose tissue itself. At that time

experimental support for this opinion could not be cited. The demonstration above that adipose tissue is capable of synthesizing glycogen during the deposition of fat, the finding that this tissue is able to break down glycogen, and the discovery of nervous and hormonal regulatory systems within the fatty tissue itself point clearly to the conclusion that adipose tissue fills more than a merely passive role and constitutes in fact a regulated organ with a definite role to play in the metabolism of carbohydrates and of fat. It is of interest to note, therefore, that a new conception of the mode of formation and role of adipose tissue has also been developed recently in a purely anatomical approach [Wassermann, 1929; Hausberger, 1937; Wells, 1940].

It is evident that a relationship exists between the experiments described above and the problem of obesity.

A further point remains to be discussed. The glycogen in adipose tissue has been shown to be subject to the influence of different factors which are specific up to a certain point for this glycogen. Adrenaline, for instance, which at first affects muscle glycogen and then liver glycogen, has no effect at all on adipose-tissue glycogen. Strychnine again, which causes a severe diminution of muscle glycogen, is without effect on adipose-tissue glycogen. Certain bacterial endotoxins, which are without effect on muscle glycogen, exert a marked effect on adipose-tissue glycogen but a relatively weak effect on liver glycogen. Thyroxin causes a sharp diminution of liver glycogen and a relatively small diminution of muscle glycogen, but may even induce a primary accumulation of the glycogen in adipose tissue. Adrenalectomy under favourable conditions need cause only a slight drop in the glycogen contents of liver and muscle, but almost always renders accumulation of glycogen in adipose tissue impossible. The specific regulation of cardiac glycogen has been dealt with in an earlier paper [Stein & Wertheimer, 1940].

SUMMARY

When rats, which have been starved are placed on a diet rich in carbohydrates, glycogen occurs in their adipose tissue in the first days of recovery. The amount may reach 1%. This adipose-tissue glycogen resembles liver glycogen in all physical and chemical properties so far investigated.

Glycogen regularly occurs in adipose tissue only under defined conditions, i.e. only when prolonged starvation is succeeded by a diet rich in carbohydrates. Carbohydrates other than glycogen are present in adipose tissue under the same conditions in only minimal quantities, if at all.

The deposition of glycogen in adipose tissue depends on the nature of the recovery diet. A diet of 70% carbohydrate, 20% protein and 10% fat is roughly optimal. If the dietary percentage of protein or fat is increased at the expense of carbohydrate, or if the amount of protein is decreased and an excess of carbohydrate is given, the amount of glycogen deposited is lessened.

In the brown interscapular fat, deposition of glycogen is particularly rapid and large, and comparable in rate and amount with its deposition in liver.

The deposition of fat in adipose tissue is already apparent 1 day after the beginning of recovery feeding and approaches a maximum after 4 days, i.e. when glycogen disappears from the adipose tissue. After this time glycogen occurs in the adipose tissue only irregularly and in very slight amounts, if at all, and deposition of fat in the same tissue is minimal. Deposition of glycogen and fat in the adipose tissues of guinea-pigs and rabbits is less regular, smaller in order of magnitude and shows a different time curve to that in rats.

Brief but repeated massage of groin adipose tissue during recovery feeding leads to a lessened deposition of glycogen and fat on the massaged side.

Bacterial endotoxins of the *Salmonella* group prevent deposition of glycogen and markedly inhibit deposition of fat in adipose tissue during recovery feeding. Muscle glycogen is not affected under similar conditions, and liver glycogen is only slightly diminished.

Cramp-inducing doses of strychnine which cause a marked diminution of muscle glycogen do not affect the glycogen content of adipose tissue.

Deposition of adipose-tissue glycogen in thyreotoxic rats during recovery feeding is enhanced, takes place earlier and diminishes sooner than in normal animals.

Adipose tissue of thyreotoxic rats consumes glycogen more rapidly than that of normal rats. Deposition of fat in adipose tissue of thyreotoxic rats is marked in the early stages of recovery feeding when glycogen is deposited, but ceases at a low level when the glycogen values approach zero.

Insulin and adrenaline do not influence glycogen deposition in adipose tissue.

Adrenalectomized male rats at death contain only minimal amounts of peripheral fat irrespective of weight loss. After partial fasting followed by recovery feeding on high carbohydrate diet the deposition of glycogen and fat in adipose tissue is generally negligible.

The differences between glycogen deposition in liver and adipose tissue were particularly large when the adrenalectomized rats received injections of NaCl and NaHCO_3 . The deposition of glycogen in adipose tissue was induced by administration of fresh cattle adrenal extract but not of desoxycorticosterone acetate (doca).

It is concluded (1) that adipose tissue can synthesize glycogen, (2) that the glycogen metabolism of adipose tissue is specifically regulated, (3) that adipose tissue can probably effect the conversion of carbohydrate into fat, and (4) that adipose tissue thus appears to play a more active part in carbohydrate-fat metabolism than has hitherto been assumed.

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OVULATION IN THE RABBIT AFTER DESTRUCTION OF THE GREATER SUPERFICIAL PETROSAL NERVES¹

By MARTHE VOGT

*From the Pharmacology Laboratory, Cambridge, and the Pharmacological
Laboratory, the College of the Pharmaceutical Society*

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IN the rodents, special stimuli are required for the secretion, by the pituitary gland, of the principles which, in the rat, cause persistence of the corpora lutea and, in the rabbit, lead to ovulation and luteinization of the follicles. Under physiological conditions these stimuli are provided by the act of mating. If mating takes place with a sterile male, the effect on the pituitary is unimpaired, and pseudopregnancy takes the place of normal pregnancy. Evidence for the nervous control of the secretion of gonadotropic hormones by the anterior lobe has been obtained both in the rat and in the rabbit: in the rat, pseudopregnancy following sterile mating is *prevented* by local anaesthesia of the cervix uteri, though the vaginal plug is normally deposited by the male [Vogt, 1933]; in the rabbit, on the other hand, electrical [Marshall & Verney, 1936] or chemical [Marshall, Verney & Vogt, 1939] stimulation of the central nervous system is capable of *producing* ovulation.

Much work has been carried out in order to trace the nervous pathway which carries the secretory impulses to the anterior lobe, but no definite conclusion has, so far, been reached. The anatomical data are not very helpful: most of the fibres known to reach the glandular part of the pituitary come from the carotid plexus and have been assumed to be of sympathetic origin. Extirpation of the cervical sympathetic, however, though interfering with pseudopregnancy in the rat produced by weak stimuli [Vogt, 1931; Haterius, 1933], does not interfere with the same phenomenon elicited by sterile mating or with ovulation in the rabbit

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[Vogt, 1933. extirpation of the entire cervical sympathetic and the stellate ganglia; Hinsey & Markee, 1933. section of the cervical sympathetic]. Two alternatives remain, as already pointed out by Hinsey & Markee [1933]:

(a) The large bundle of fibres running from the supra-optic nuclei through the pituitary stalk into the posterior lobe might carry impulses which could be transmitted by a hitherto unknown mechanism to the glandular part of the pituitary. A direct innervation of the anterior lobe by the supra-optico-hypophyseal tract, if it exists, has so far not been detected anatomically, only very few fibres of that tract having been seen to enter the anterior lobe [Hair, 1938; Brooks & Gersh, 1938]. The role of the hypothalamus has been tested with physiological methods by a number of investigators, who either stimulated this region electrically [Harris, 1937; Haterius & Derbyshire, 1937], or destroyed the pituitary stalk [Harris, 1937; Brooks, 1938; Dempsey & Uotila, 1940]. The difficulty, however, in limiting either stimulation or damage to, for instance, the hypothalamo-hypophyseal tracts is so great that the interpretation of the results meets with considerable difficulties. The general atrophy of the sexual organs which follows section of the stalk in the rabbit [Harris, 1937] provides another complicating factor. In the rat, Dempsey & Uotila [1940] reported normal pregnancy after section of the pituitary stalk.

(b) Parasympathetic fibres running in the carotid plexus might innervate the anterior lobe. Such fibres were discovered in the greater superficial petrosal nerve of the cat and the monkey by Cobb & Finesinger [1932] and Chorobski & Penfield [1932]. They leave the petrosal nerve for the pericarotid plexus at an anastomosis with the internal carotid nerve before the petrosal enters the Vidian canal. They were shown to carry vasodilator impulses to the pial vessels. The frequent association of vasodilator and secretory fibres would favour the hypothesis that the fibres entering the anterior lobe from the carotid plexus might contain secretory fibres originating in the greater superficial petrosal nerve. In the rat, Zacharias [1941] has actually described fibres running from the junction of the superficial and deep petrosal nerves to the capsule and parenchyma of the anterior lobe. This bundle may, in his opinion, be a pathway for impulses from the superficial petrosal nerve to the hypophysis.

The present work was done in order to test this second alternative. Ovulation in the mated oestrus rabbit was used as a test for the integrity of the innervation of the anterior lobe. Bilateral destruction of the petrosal nerve at its passage through the geniculate ganglion was carried

out and the effect of this operation on ovulation observed. While the experiments were in progress, it was found that Hair & Mezen [1939] had already attacked the same problem in a different manner. Finding it impracticable to keep in good condition animals in which the petrosal nerve had been destroyed by an operation through the ear, Hair & Mezen discovered that by avulsion of the facial seized in a clamp at the stylo mastoid foramen the geniculate ganglion was frequently removed as well: it was found attached to the facial when the nerve was examined microscopically. Seven out of eighteen rabbits in which this operation had been successfully carried out on both sides ovulated. The conclusion drawn was that the greater superficial petrosal nerve was not innervating the anterior lobe. This conclusion, however, is not quite convincing on anatomical grounds. The fibres which run via the petrosal nerve into the carotid plexus cross the dorsal surface of the facial as part of the *nervus intermedius* and pass *along* the geniculate ganglion without being interrupted in it. Since Hair & Mezen do not report post-mortem examination of the skulls of the operated rabbits, it is conceivable that their procedure which tore away the facial and the cells of the geniculate ganglion did not sever the motor part of the petrosal nerve. The technique I have used is not open to the same criticism and will therefore be described in detail although it is far less simple than that of the American authors.

METHODS

Plan of experiments

Young adult does were used which were kept in separate hutches. Except for one animal in which the operations were performed in November, the rabbits were all operated on between February and May, the time of year when normal isolated does are most likely to be on heat [Hammond & Marshall, 1925]. As soon as the operations had been completed on both sides, mating was attempted at frequent intervals. It will be seen later that it took periods varying from 3 days to over 5 months till the does had sufficiently recovered from the operation to accept the buck. This point often coincided with the moment at which they recovered their preoperative weight; the absence of oestrus was, therefore, probably due to the damage from the severe operation, especially to the ensuing malnutrition (see below), and not to any specific effect of the destruction of the petrosal nerve.

Although no serious doubt usually arises from the behaviour of normal rabbits whether mating has taken place or not, as an additional

precaution a vaginal smear was taken after copulation and the presence or absence in it of spermatozoa was ascertained; the latter were always found when copulation was believed to have been successful.

Surgical procedure

The rabbits were anaesthetized with ether after a subcutaneous injection of 5 mg. atropine sulphate. Strict asepsis was observed in all the operations. Each side was operated on separately. The best results were obtained if the interval between the two operations was no shorter than 3 weeks.

The superficial petrosal nerve of the rabbit is enclosed in a bony channel of the petrous bone during its entire course from the geniculate ganglion to the internal carotid artery and is, therefore, only accessible within the bone. Two modes of approach to it proved feasible. In the one, a horizontal incision was made through the skin across the bony part of the external acoustic meatus, and access gained to the tympanic cavity by drilling first through the lateral and then through the mesial surface of the auditory meatus; the facial was traced and destroyed up to its origin from the brain by means of a small dental drill. With the facial the intermediate nerve and, therefore, the petrosal was severed from the brain. In spite of definite advantages of this method—the branches of the external carotid and the superior laryngeal nerve are not endangered in that approach—it was later replaced by a ventral exposure which required less drilling through bone and did not necessarily involve complete destruction of the seventh nerve. In this method the animal was tied on its back, a midline incision made through the skin of the neck, and the ventral surface of the tympanic bulla exposed in the following manner. Submaxillary and parotid glands were pushed laterally and kept there by a weighted ligature; the tendon of the digastric muscle was cut and its posterior portion retracted caudally by another weighted ligature; a third ligature was looped round the external carotid and external maxillary arteries and weighted so as to pull them orally. The bone could then be reached by dissecting bluntly between the tendons of the stylohyoideus minor and styloglossus on the one hand, and the deep muscles covering the vertebral column on the other hand. A hole about 3 mm. in diameter was made with a dental drill in the ventral surface of the tympanic bulla and widened orally. The glossy tendon of the musculus tensor tympani became visible through the hole, and since the superficial petrosal nerve runs dorsally to this muscle, a useful landmark for the drilling was thus provided. A headlamp greatly facilitated orientation at this stage. The drill (No. 6 or 7 of the dentist's scale) was inserted into

out and the effect of this operation on ovulation observed. While the experiments were in progress, it was found that Hair & Mezen [1939] had already attacked the same problem in a different manner. Finding it impracticable to keep in good condition animals in which the petrosal nerve had been destroyed by an operation through the ear, Hair & Mezen discovered that by avulsion of the facial seized in a clamp at the stylo mastoid foramen the geniculate ganglion was frequently removed as well: it was found attached to the facial when the nerve was examined microscopically. Seven out of eighteen rabbits in which this operation had been successfully carried out on both sides ovulated. The conclusion drawn was that the greater superficial petrosal nerve was not innervating the anterior lobe. This conclusion, however, is not quite convincing on anatomical grounds. The fibres which run via the petrosal nerve into the carotid plexus cross the dorsal surface of the facial as part of the nervus intermedius and pass *along* the geniculate ganglion without being interrupted in it. Since Hair & Mezen do not report post-mortem examination of the skulls of the operated rabbits, it is conceivable that their procedure which tore away the facial and the cells of the geniculate ganglion did not sever the motor part of the petrosal nerve. The technique I have used is not open to the same criticism and will therefore be described in detail although it is far less simple than that of the American authors.

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Although no serious doubt usually arises from the behaviour of normal rabbits whether mating has taken place or not, as an additional

Post-mortem examinations

The ovaries were inspected 2 or 3 days after mating by exploratory laparotomy under ether anaesthesia; if ovulation had occurred, the rabbit was killed and the posterior half of the skull decalcified in a mixture of formalin and nitric acid; paraffin blocks were made of the site of operation and serial frontal sections through these blocks were stained and examined microscopically. This control was found necessary, since scar formation made the macroscopical interpretation even of decalcified skulls uncertain.

RESULTS

Six rabbits which survived the operation on both sides made a sufficiently good recovery to come on heat. When mated, all of them ovulated; between three and five corpora lutea were found in each ovary.

Microscopical examination of the skulls revealed complete destruction of both petrosal nerves in the neighbourhood of the geniculate ganglion in four of the animals. The remaining two had to be discarded since the right petrosal nerve was found intact in the first, and damaged instead of destroyed in the second.

In the four successfully operated rabbits intervals of 3, 25, 74 and 162 days elapsed between the second operation and the occurrence of oestrus. Three of them behaved uniformly and ovulated after the first mating; the fourth animal, oestrus appearing on the 25th post-operative day, did not ovulate after having been served by the buck. In order to test whether its ovaries were normal and the failure to ovulate was caused by lack of pituitary secretion, this animal was injected intravenously with a saline extract of 4 mg. of a powder prepared from rabbit's anterior lobe by dehydrating the tissue with acetone and evaporating the excess solvent in a desiccator: normal ovulation and formation of corpora lutea were observed at a laparotomy 3 days later. In order now to decide whether the absence of ovulation after mating was to be attributed to the unknown accidental cause which produces a certain percentage of unfertile matings in the normal rabbit or to a specific effect of the section of the petrosal nerves, use was made of Hammond & Marshall's [1925] observation that mating is always fertile on the first days following either parturition or pseudopregnancy. The pseudopregnancy which had resulted from the injection of anterior lobe extract was allowed to pass off, and the rabbit was mated for a second time 21 days after injection of the extract. This time ovulation ensued in both ovaries. The failure to ovulate on the first occasion could, therefore, not be interpreted as a proof of innervation of the anterior lobe by the petrosal nerve.

the bony groove containing the tensor tympani and directed dorso-medially. A channel was drilled in the bone till the inner surface of the skull was reached; this channel usually destroyed part of the facial at the genu, the whole of the geniculate ganglion and the origin of the petrosal nerve. Occasionally, if the hole was made a little more orally, a large part of the facial remained intact and the petrosal nerve only was completely interrupted.

When the drill entered the skull, it frequently but not regularly caused venous haemorrhage, which, however, usually had no serious consequences. As soon as the bleeding had stopped, the wound was closed by suturing the fascia of the neck and the incision through the skin.

Two serious difficulties are encountered during this operation. In the rabbit, the superior laryngeal nerve runs so near the tympanic bulla that dissection and traction in that region often causes reflex respiratory standstill which is frequently fatal. Attempts at reducing this danger by using some local anaesthetic at the site of operation made matters worse instead of better; the only solution was very cautious handling of all the structures surrounding the bulla and constant observation of the effect on the rabbit's respiration. The other danger is damage to the labyrinth. Unilateral destruction, though not lethal, affects the animal's posture so violently that its food uptake is seriously hampered; bilateral damage leads to muscular atony and eventually to death. If the injury is only slight, no serious impairment of the rabbit's general health need result, although a longer interval than usual between the first and second operation is required.

Aftercare of the animals

Even if the operations are carried out without damage to the labyrinth, the food uptake of the rabbits suffers from the paresis or paralysis of the facial. The buccinator muscle is often paralysed, and plugs of unswallowed food may remain and decay in the animals' mouth. Moreover, damage to the chorda tympani must lead to decreased production of saliva, and the destruction of the petrosal nerve itself is bound to cause loss of secretion of the glands of the palate and therefore to affect the animals' feeding. It is not always possible to compensate for these deficiencies by stomach tube feeding, and some animals were lost from mere inanition after the second operation. On the other hand, it is well known that malnutrition will prevent follicular growth and occurrence of heat in does; it is, therefore, essential to make every effort to induce the operated rabbits to eat as much as possible.

THE ACTION OF CURARE ON THE RESPIRATORY CENTRE

By J. FEGLER

*From the Institute of General Pathology, University of Cracow, and the
Laboratoire de Physiologie Générale, Sorbonne, Paris*

(Received 14 September 1941)

AN investigation into the action of curare on the respiratory centre which was being conducted by the author at Cracow in 1939 was interrupted by the outbreak of war. The work was resumed, however, at a later date when, by means of facilities generously provided by Prof. L. Lapicque, the author was able to extend this study to include measurements with Lapicque's method of the summation time of the respiratory centre, of the rheobasic voltage and of chronaxie for stimulation of the central ends of the sciatic and vagal nerves.

The experiments which are to be described in the present paper do not yield sufficient data to make a complete analysis of the problem, but the preliminary results appear to be of sufficient interest to warrant giving a short account of them.

METHODS

Dogs, 6-10 kg: in weight, were anaesthetized with morphine and chloralose. Tracheotomy was then performed and an oxygen cylinder connected to the tracheal cannula; from this point on, throughout the whole observation period, the animals breathed pure oxygen gas. During the initial phase of each experiment the animal's own respiration provided sufficient ventilation, but after the injection of curare, when there was a partial inhibition of respiration, it was necessary to introduce artificial respiration. This was done by rhythmically inflating the lungs with oxygen (under positive pressure) in step with the animal's respirations, so that the amplitude was increased sufficiently to make the oxygen

DISCUSSION

In spite of the evidence showing that the production of pseudo-pregnancy in the rat and ovulation in the rabbit requires nervous stimuli to the anterior lobe of the pituitary, the pathway carrying these impulses is not established. The hypothesis that the superficial petrosal nerve which carries vasodilator fibres to the pia also transmits the secretory impulses needed for ovulation has been disproved by the experiments reported in this paper. Unless hitherto unknown fibres from other cranial nerves join in the formation of the pericarotid plexus, these results provide indirect support for the view, held by many experimenters but still open to doubt, that the hypothalamus conveys, either directly or via the posterior lobe, impulses to the glandular part of the hypophysis.

SUMMARY

Four rabbits in which the greater superficial petrosal nerve had been severed bilaterally, nevertheless ovulated normally when on heat and mated.

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The experiments which are to be described in the present paper do not yield sufficient data to make a complete analysis of the problem, but the preliminary results appear to be of sufficient interest to warrant giving a short account of them.

METHODS

Dogs, 6-10 kg: in weight, were anaesthetized with morphine and chloralose. Tracheotomy was then performed and an oxygen cylinder connected to the tracheal cannula; from this point on, throughout the whole observation period, the animals breathed pure oxygen gas. During the initial phase of each experiment the animal's own respiration provided sufficient ventilation, but after the injection of curare, when there was a partial inhibition of respiration, it was necessary to introduce artificial respiration. This was done by rhythmically inflating the lungs with oxygen (under positive pressure) in step with the animal's respiration so that the ventilation was increased sufficiently to make the oxygen

available to the animal in the same amounts as it had been before the injection. For the observations of the respiratory responses to reflex stimuli it was necessary to interrupt artificial respiration for brief periods, but it must be stressed that these periods of reduced ventilation were always brief, and that it is improbable that any significant degree of anoxia developed during the course of the experiments. Indeed, previous experience [Chauchard, Chauchard & Fegler, 1940; Fegler, Chauchard & Chauchard, 1940] goes to show that the effects of anoxia on the respiratory centre are quite unlike those which are ascribed to curare in the present report.

In the preliminary experiments performed at Cracow, the central end of the cut sciatic nerve was stimulated with a faradic current both before the injection of curare and later, during the period of respiratory inhibition. In Paris these observations were repeated, and the effects of curare on the respiratory responses to stimulation of the central end of the cut cervical vagus nerve were studied as well. In these experiments the other vagus nerve was left intact. The second group of experiments included measurements of chronaxie (by Lapique's method), some of which are given in Tables 1 and 2.

For the sciatic nerve the minimal stimulus required to produce a perceptible shortening of the respiratory interval was taken as the threshold stimulus (excitatory effect). Conversely, the threshold stimulus for the vagal nerve was the minimal stimulus required to produce a minimal apnoea (inhibitory effect).

Two preparations of curare were tested: the first was a 1% solution of Merck's curare (used in the Cracow experiments), and the second was a preparation made up from one fraction of the curare known as 'bambou' [Lapique & Lapique, 1940], which was kindly supplied to the author by Prof. Lapique. The 'bambou' fraction was prepared for injection by precipitating it with alcohol, and then dissolving the precipitate in physiological saline to a concentration of 3.5%. For the purposes of the investigation 2-5 c.c. of either Merck curare or the 'bambou' fraction were sufficient, and the range of doses used was within these limits.

RESULTS

Respiratory inhibition by curare. The intravenous injection of 2-5 c.c. of either of the curare preparations produced, after a short interval of respiratory excitation, a decrease of respiratory frequency and amplitude, this inhibition being a progressive one which eventually resulted in the cessation of the animal's respiratory movements. In the initial phase

of the respiratory inhibition thus induced, faradic stimulation of the central end of the sciatic nerve caused an increase in respiratory frequency and amplitude. At a later stage of the inhibition, however, similar stimulations failed to alter either the frequency or depth of respiration. When respiratory movements finally ceased, the animals continued to be unresponsive to sciatic stimulation, but at this point in the experiments faradic stimulation of the phrenic nerve in a peripheral position still caused contraction of the diaphragm. Furthermore, this response to phrenic nerve stimulation was only prevented by the administration of a larger dose of curare than that required to cause respiratory arrest.

These experiments seemed to indicate that the respiratory inhibition which is produced by curare injections is in the first instance due to an action on the respiratory centre, that is, it acts by creating a block across the apices of the reflex arc.

This interpretation might also be extended to the effects produced by curare on certain cardiac reflexes. As a corollary to the present subject of investigation the cardiac responses to reflex vagal stimulation were observed, and it was noted that curare (dose 2-5 c.c.) progressively diminished the effectiveness of the stimulation in producing cardiac arrest, although stimulation of the peripheral end of the vagus had a marked inhibitory action on the heart of the curarized animal.

Measurements of chronaxie. The results obtained with this method are illustrated by the experiments given in Tables 1 and 2. In the experiment shown in Table 1 the effect of sciatic nerve excitation was investigated. The dog, 6 kg. in weight, received 5 c.c. of a 3.5% solution of curare.

TABLE 1

	Rheobase in V.	Time of summation in sec.	Chronaxie capacity in μ F.
Right sciatic normal	6.5	7	0.02
5 min. after curare	7.5	9	0.02
15 min. after curare	5.5	10	0.06
25 min. after curare	25.0	12	0.07
40 min. after curare	30.0	17	0.08

From these results it is evident that the injection of curare produced a well-marked diminution in the excitability of the respiratory centre, this effect being indicated by the early increase in summation time and the later increase in the rheobase voltage and chronaxie.

In the experiment shown in Table 2 the effect of excitation of the central ends of the vagal nerves was studied.

TABLE 2

	Rheobase in V.	Time of summation in sec.	Chronaxie capacity in μF .
Right vagus normal	10	3	0.01
6 min. after curare	10	3	0.01
15 min. after curare	13.5	3	0.04
34 min. after curare	22	5	0.04
40 min. after curare	24	8	0.06
50 min. after curare	Complete inexcitability		

It may be seen from this experiment that the effect of curare on vagal stimulation (minimal apnoeic effect) was analogous to the effect produced on sciatic stimulation (excitatory effect).

DISCUSSION

The results of the experiments which have been described indicate that the effect of curare, in the doses used, was to cause a progressive depression of the respiratory centre, so that reflex stimuli which normally affect the respiratory frequency and amplitude, either by increasing or diminishing respiration, lose their effectiveness. The fact that an appropriate dose of curare produces not only a diminished amplitude, such as might be produced if its action were purely peripheral, but also a reduction in the frequency of respiration, indicates that the drug does act centrally, at least in its initial phase of action. That is why, in the experiments in which the measurements of chronaxie were made, the influence of curare on the reflexly produced changes of respiratory rhythm, rather than on changes of respiratory amplitude, was investigated. With regard to the interpretation of these particular experiments, it is demonstrated by Lapicque's work that an increase in summation time denotes a loss of central excitability in the reflex arc.

Harvey [1940], while investigating the action of quinine methochloride, found a complete analogy between the action of this drug and that of curarine; but he states as well that the compound has no central action. Harvey's experiments might be interpreted differently, however. It is known that curare does not alter the character of the discharges from the respiratory centre along the phrenic nerve, and it may be that the amplitude and the rhythm of the discharges during the active period of the centre (inspiration) are unchanged when curare is administered. It is still possible, however, that the frequency of the active periods of the respiratory centre is less, and this is more readily shown by recording the respiration itself. Unfortunately, the data given in Harvey's paper do not make it clear whether or not he took cognisance of the actual

respiration rate before and after injection of the compound in question, and the fact that in his experiments the character of the discharges along the phrenic nerve during active periods of the respiratory centre was not altered, does not preclude the possibility that the frequency with which those active periods occurred may have been lessened.

The most probable explanation of the observed phenomena is that the initial respiratory inhibition produced by curare is due to an interruption of respiratory reflexes, that is, the sensory impulses are blocked so that the respiratory centre is released both from excitatory and inhibitory impulses, and its periods of activity then depend upon its own basal rhythm and possibly on the chemical changes of the blood. Released from sensory nerve influences, the periods of activity would be diminished, but, during activity, the discharge of impulses along the motor nerve would not necessarily be altered. It is possible that the brief period of excitation, following the injection of curare, depends upon the release of the centre from inhibitory sensory impulses, and that for a short period the centre is subject to predominantly excitatory impulses, which in turn are blocked and the inhibitory phase supervenes. It may be, however, that the excitatory effect depends upon some impurity contained in the curare preparations used. It is at most a transitory effect and need not concern us here.

The thesis elaborated here that curare alters the rhythm of the respiratory centre by blocking the transmission of sensory impulses through the respiratory centre is in line with accepted views on the action of curare on the neuromuscular junction and with the conclusion reached by Brown & Feldberg [1936] that the drug blocks the transmission of nerve impulses across sympathetic ganglia. The experiments reported now indicate that central nervous structures are affected in the same way, the only difference being that, with the doses of curare which were used in this investigation, the onset of the central block, of which the initial respiratory inhibition is symptomatic, is more rapid than the onset of the peripheral effect which produces the final cessation of respiratory movements. In each case, however, the mechanism is essentially the same, and depends upon the affinity of the drug for the synaptic structures of the nervous system.

SUMMARY

1. Intravenous injection of curare in anaesthetized dogs produces a short period of excitation, followed by inhibition of respiration as shown by a decrease in the respiratory frequency and amplitude. The respi-

ratory inhibition is progressive, and eventually results in the cessation of respiratory movements.

2. During the period of respiratory inhibition, sensory stimuli which normally augment (central sciatic stimulation) and those which normally diminish (central vagal stimulation) respiratory amplitude and frequency become completely ineffective in producing such changes, although stimulation of the phrenic nerve still produces contraction of the diaphragm.

3. It has also been found that curare produces an increase of the summation time and of the chronaxie and rheobase voltage for stimulation of the central ends of the sciatic and vagal nerves.

4. The slowing of respiratory rhythm, together with the progressive depression of the responses to reflex stimuli which either excite or inhibit the respiration, seems to prove that curare influences the respiratory centre before the full development of its peripheral action.

The author wishes to express his gratitude to Prof. L. Lapicque for the facilities which were placed at his disposal at the Laboratoire de Physiologie Générale where the greater part of this research was carried out.

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THE INFLUENCE OF A DEFICIENCY OF VITAMIN B₁ AND OF RIBOFLAVIN ON THE REPRODUCTION OF THE RAT

By KATHARINE HOPE COWARD,
BARBARA GWYNNETH EMILY MORGAN
AND LETITIA WALLER

From the College of the Pharmaceutical Society, London

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EVANS & BISHOP [1922] found that 'vitamin B' (not then recognized as comprising several substances) was necessary for the normal physiological functions of the female sex organs. ~~In its absence~~ leucocytes and nucleated epithelial cells were formed day after day in the vagina but no keratinized cells. Macy, Outhouse, Graham & Long [1927] found that on a partial deficiency of vitamin B sexual maturity was delayed, reproduction took place but lactation was uniformly unsuccessful. Lately, Coward & Morgan [1941] have investigated the influence of a vitamin B₁ deficiency in the diet on the vaginal contents of mature virgin rats with a view to using it as a criterion in the determination of vitamin B₁ in food substances. The rats used in this investigation had suffered a deficiency of vitamin B₁ to various extents both in the amounts of vitamin B₁ given and in the lengths of the periods of deficiency. These animals were therefore used to study the effect of the deficiency on the rats' powers of reproduction. In addition, the influence of riboflavin deficiency on the vaginal contents and reproduction has been investigated in a number of the rats which had been employed by Lindholm [1938] in his work on the determination of riboflavin.

VITAMIN B₁ DEFICIENCY

Methods

The rats had first been given a diet deficient only (as far as was known) in vitamin B₁, and when no cornified cells had appeared in the

vagina of any rat for a period of 10 days it was assigned to one of six groups in which the rats were receiving daily doses of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.1 g. respectively of an adsorbate containing 200 I.U. of vitamin B₁ per g. When a rat had produced three cycles of cornified cells in the vagina, the dose of vitamin B₁ was withheld until it had again exhibited no cornified cells in the vagina for 10 days. It then received the same dose as before until it had produced three more cycles, when the whole process was repeated yet again. It was clearly shown that the response (time taken to produce three cycles) was graded to the dose of vitamin B₁ given, and that the rats became increasingly sensitive to successive doses of equal amounts of vitamin B₁. The basal diet given to these rats throughout the experiment consisted of:

Cascinogen (physiological caseinate, Glaxo)	15 parts
Dextrinized rice starch	70 "
Agar-agar	2 "
Salt mixture (Steenbock's 40)	4 "
Dried yeast, autoclaved at 120° C. for 6 hr.	25 "
Cod-liver oil, 5 drops of a good sample per rat twice a week	

Fresh tap water was supplied daily, and the cages were provided with grids of $\frac{1}{2}$ in. mesh to prevent the rats from having access to their faeces. Six or seven rats were kept together in a large cage during the preparatory (no vitamin B₁) periods, but each one had a cage to itself during its dosing period.

Results

Effect on vaginal contents

Two of the seven rats given 0.01 g. (2 I.U. of vitamin B₁) of the adsorbate produced one cycle only, and then lapsed into anoestrus. The other five produced no cycles at all. Of the twelve rats given 0.02 g., two produced no cycles, one produced one cycle only, seven produced two series of three cycles and two produced three series of three cycles. Of the fourteen given 0.03 g., one produced no cycle, one produced one cycle, one produced one series of three cycles and later a series of two cycles, the rest (eleven) produced three series of three cycles each. All of the thirteen rats given 0.04 g., the sixteen rats given 0.05 g., an extra group of eleven rats given 0.05 g. with 16 days of no cornified cells in the intervals between periods of dosing, and thirteen rats given 0.1 g. adsorbate, produced three series of three cycles each (except rat 2515 which did not complete its third series; see also Table 1 for this rat's later failure to reproduce). Thus the rhythm of cycles in the vaginal contents was restored by giving vitamin B₁, and the response (time

taken to produce three cycles) was graded to the dose of vitamin B₁ given. Moreover, the rats became increasingly sensitive to successive treatments with equal doses of vitamin B₁ [Coward & Morgan, 1941].

Effect on reproduction

Rats which had completed three series of three cycles in the first part of this experiment were then subjected to a test of reproduction. Some were still given the vitamin B₁-free diet but with the addition of 1% of the adsorbate containing 200 I.U. of vitamin B₁ per g., each rat thus receiving from 20 to 30 units per day. Other rats were given the complete diet of our rat colony. This consisted of:

Whole yellow maize (ground)	65	parts
Whole wheat (ground)	20	"
Casein (light white, B.D.H.)	9	"
Dried yeast	5	"
Dried whole milk	20	"
Calcium carbonate	0.5	"
Sodium chloride	0.5	"
Potassium iodide	0.0025	parts
Ferric citrate	0.1	"

The results may be seen in Tables 1 and 2. It is evident that the fertility of the rats which had received the lower doses of vitamin B₁ was seriously impaired, whereas that of the rats which had received the higher doses was not (Table 1). It is also evident that large doses of vitamin B₁ in addition to the vitamin B₁-free diet did not make the diet adequate for reproduction (Table 2). This indicated very strongly that the failure of the rats to produce young was not due to a deficiency of vitamin B₁ only, if indeed at all. The addition of wheat-germ oil was then tried but failed to improve the fertility of these rats (Table 2). Seven of them were then given the complete diet; three of them produced and weaned healthy litters, but three did not; one which appeared to be sterile after 33 days on the diet was killed and foetuses were found in both arms of the uterus. It must therefore be concluded that the B₁-free diet was lacking not only in vitamin B₁ but in some other factor necessary for reproduction, which was not vitamin E. (We have not attempted to determine what this factor is.) The sterility of the rats which had received only small doses of vitamin B₁ in periods preceding feeding on the complete diet was not explained then by the shortage of B₁, though it was possible that the greater length of time during which these rats had suffered a partial deficiency might have contributed to the deleterious effect. In Table 2 has been inserted the length of time during which each rat had been deprived of a possible unknown factor, i.e. the

time composed partly of complete deficiency and partly of partial deficiency of vitamin B₁. It is evident that there is a close correlation between this length of time and the sterility of the rats. Only one rat, 2348, whose period of deficiency had been as long as 178 days, was fertile when given a complete diet, and one rat, 2935, whose period of deficiency had been as short as 109 days, was sterile.

TABLE 1. Reproduction after vitamin B₁ deficiency

Previous daily dose of vitamin B ₁ adsorbate g.	Rat	Period on partial deficiency of vitamin B ₁ days	Wt. of adsorbate given during this period g.	Av. daily intake of adsorbate g.	Wt. of rat when first mated g.	Fertility when given complete diet	
						Litter weaned	Av. wt. at 3 weeks
0.02	2382	191	3.04	0.016	170	0	
	2529	191	2.86	0.015	147	0	
	2351	192	2.84	0.015	161	0	
	2921	184	2.68	0.015	144	0	
	2348	178	2.66	0.015	142	1♂ 1♀	33
	2860	183	2.82	0.015	138	0	
	2944	181	2.80	0.015	165	0	
0.03	2380	187	4.62	0.025	140	0	
	2362	111	1.95	0.018	174	3♀	31
	167	114	1.41	0.012	165	3♂ 3♀	38
0.04	2935	109	2.32	0.021	196	0	
	2915	183	5.60	0.031	169	0	
	2919	95	1.72	0.018	164	4♂	46
	2511	108	2.00	0.019	178	5♂ 4♀	33
0.05	2936	91	2.75	0.030	194	3♂ 2♀	40
	2911	100	3.45	0.035	182	2♂ 2♀	38
	2525	107	3.35	0.031	190	5♂ 2♀	37
	42	106	3.10	0.029	174	4♂ 3♀	31
	166	107	2.35	0.022	190	1♂ 3♀	55
	9965	114	2.95	0.026	158	2♂ 5♀	24
0.1	154	114	3.60	0.032	158	4♂ 3♀	29
	165	125	5.10	0.041	180	2♂	40
	9805	125	4.60	0.037	187	6♂ 4♀	27
	2530	107	5.10	0.048	192	4♂ 5♀	27
	2515	177	13.30	0.075	157	0	
	2464	101	5.40	0.053	193	3♂ 6♀	32
	515	109	6.30	0.058	166	2♂ 5♀	29

Thus it is probable that our vitamin B₁-free diet was deficient in at least two factors, vitamin B₁ and an unidentified factor. It is, however, quite evident that a partial deficiency of vitamin B₁, plus a partial or complete deficiency of some unknown factor for periods up to about 120 days, does not do irreparable damage to the reproductive functions of the rat. It is also evident that a more prolonged and more severe deficiency of vitamin B₁ or of some hitherto unrecognized factor does damage which is not repaired by giving a complete diet.

TABLE 2. Reproduction after vitamin B₁ deficiency

Previous daily dose of vitamin B ₁ adsorbate μ	Part on partial deficiency of vitamin B ₁ days	Wt. of adsorbate given during this period μ	Av. daily intake of adsorbate μ	Wt. of rat when first mated μ	Fertility when given			Av. wt. at 3 weeks μ
					(a) B ₁ -free diet + 1% adsorbate (200 units per μ)	(a) +0.1 μ wheat germ oil daily	(a) +killed by \varnothing on 2nd day	
0.03	166	2.07	0.018	166	+	+	+	—
	2178	2.46	0.020	170	0	—	—	—
	2033	3.00	0.023	171	0	—	—	—
	2385	2.10	0.019	136	+	—	—	—
	2	2.80	0.023	160	+	—	—	—
0.01	2483	2.44	0.022	180	+	—	—	—
	2010	2.80	0.024	182	+	—	—	—
	2465	2.72	0.023	169	+	—	—	—
	2485	2.12	0.023	165	+	—	—	—
	2523	2.36	0.023	170	+	—	—	—
0.05	2551	2.30	0.031	176	+	—	—	—
	9733	4.15	0.030	161	+	—	—	—
	211	4.20	0.033	168	+	—	—	—
	425	2.90	0.025	170	+	—	—	—
	2005	2.90	0.021	178	+	—	—	—
0.1	2408	5.60	0.032	165	+	—	—	—
	2636	0.20	0.018	175	+	—	—	—
	8	7.80	0.071	170	+	—	—	—
	2051	7.70	0.055	160	+	—	—	—
	63	0.70	0.040	170	+	—	—	—
0.075 Int. St.	9723	13.00	0.076	165	+	—	—	—
	2381	3.20*	0.021*	180	+	—	—	—
	2045	3.18	0.018	175	+	—	—	—
	2038	2.63	0.017	174	+	—	—	—
	2550	—	—	—	+	—	—	—

* The International Standard adsorbate for vitamin B₁ contained only 100 units of vitamin B₁ per μ ; therefore the amount actually given to each rat was divided by 2 in this table to make the amounts comparable in vitamin B₁ content with the amounts of adsorbate containing 200 units per μ given to the other rats.

RIBOFLAVIN DEFICIENCY

Methods

When Lindholm's work [1938] on the determination of riboflavin by the use of rats was nearing completion, it occurred to us that we could use his animals for an investigation of the influence of riboflavin deficiency on the vaginal contents and reproduction just as we were investigating the influence of vitamin B₁ deficiency [Coward & Morgan, 1941]. Only eleven of his rats were still on experiment; the rest had completed their period of dosage and had been discarded. This small number of animals however gave decisive results.

Lindholm's rats had been given a diet consisting of:

Caseinogen (physiological caseinate, Glaxo)	15%
Dextrinized rice starch	77%
Agar-agar	2%
Salt mixture (Steenbock's 40)	4%
Vitamin B ₁ powder (B.D.H., 100 I.U. per g.), i.e. about 12 I.U. per rat per day	2%
Cod-liver oil, 6 drops of a good sample per rat twice a week.	
Liver extract [Halliday & Evans, 1937] corresponding to 2 g. fresh liver daily.	

When they ceased to grow, they were distributed into six groups and given graded doses of riboflavin daily for 21 days, every rat of any one group being given the same dose. The response, increase in weight in 3 weeks, was graded to the dose of riboflavin given.

The time taken by each rat to become steady in weight is designated 'First period of riboflavin deficiency' in Table 1. The vaginal contents of each rat were first examined on the day it received the 21st dose of riboflavin and every day subsequently. Thus the weight of the rat when its vaginal contents were first examined was its weight at the end of the 21 days' dosing.

*Results**Effect on the vaginal contents*

It soon became evident that the rats which had received no riboflavin or only small doses (1-7.5 μ g. daily) were in anoestrus, while two of the three which had received large doses (30 μ g.) were producing cycles regularly. When anoestrus had lasted for 22-32 days five of the nine rats were given daily doses of 20 μ g. riboflavin, and the other four were given 10 c.c. of milk each. Eight of these nine rats produced three cycles within 15-40 days, the other rat produced one cycle only in 15 days and no more within a further 26 days. (The time required to complete three cycles did not seem to be dependent on the size of dose given in the first part of the experiment.) Hence the mechanism which regulates

TABLE 3. The influence of riboflavin deficiency in the diet of a rat on its vaginal contents and reproduction

Rat	Wt. when first given deficient diet g.	First period of riboflavin deficiency days	Wt. first given riboflavin g.	Dose of riboflavin daily for 21 days μ g.	Wt. at end of dosing period g.	Second period of riboflavin deficiency days	Second daily dose of riboflavin (or milk)	Effect on vaginal contents	Reproduction
5038	60	43	(89)	0	(78)	23 anoestrus	20	3 cycles in 12, 15, 13 days	Mated at 140 g. wt. Given complete diet—no litter within 128 days
4898	67	43	(110)	0	(110)	23 anoestrus	20	3 cycles in 4, 7, 8 days	Mated at 104 g. wt. Given complete diet—no litter within 60 days
4845	60	43	110	1	126	23 anoestrus	20	3 cycles in 8, 13, 9 days	Mated at 106 g. wt. Litter born 23 days later, but dead in 2 days. Given 1 week's rest, remated at 105 g. Given complete diet—no litter within 110 days
5017	53	43	61	1	63	32 anoestrus	10 c.c. milk	3 cycles in 4, 7, 7 days	Mated at 120 g. wt., litter born 27 days later, but dead in 23 days. Remated at 185 g. Given complete diet, litter born 28 days later, weaned 5 σ 3 g, av. wt. 31.0 g.
5015	55	28	71	2.5	77	23 anoestrus	20	1 cycle in 15 days, anoestrus for 26 days	Mated at 140 g. wt. Given complete diet, litter born 31 days later, weaned 5 σ 3 g, av. wt. 39 g.
4850	56	43	90	2.5	96	32 anoestrus	20	3 cycles in 5, 6, 1 days	Mated at 127 g. wt. Given complete diet, litter born 21 days later, weaned 3 σ 2 g, av. wt. 38.5 g.
5010	55	43	66	2.5	70	32 anoestrus	10 c.c. milk	3 cycles in 10, 14, 6 days	Mated at 163 g. wt. Given milk ad lib., litter born 24 days later, dead in 7 days. Given 1 week's rest, remated at 160 g. Given complete diet, litter born 28 days later, weaned 3 σ 2 g, av. wt. 15 g.
4783	59	53	115	7.5	120	22 anoestrus	10 c.c. milk	3 cycles in 11, 12, 11 days	Mated at 185 g. wt. Given milk ad lib., litter born 37 days later, weaned 3 σ 4 g, av. wt. 28 g.
1801	62	50	100	30	130	4 cycles in 7, 7, 15 days resp., then 26 days anoestrus	—	—	Mated at 140 g. wt. Given milk ad lib., born 25 days later, probably a litter. Given 1 week's rest, remated at 187 g. wt. Given complete diet, litter born 20 days later, weaned 1 σ 7 g, av. wt. 38 g.
5018	62	47	100	30	135	3 cycles in 6, 14, 21 days resp., then 21 days anoestrus	—	—	Mated at 150 g. wt. Given milk ad lib., litter born 22 days later, weaned 2 σ 6 g, av. wt. 50 g.
5175	63	37	90	30	113	24 anoestrus	10 c.c. milk	3 cycles in 8, 10, 10 days	Mated at 160 g. wt. Given milk ad lib., litter born 23 days later, dead in 2 days. Remated 1 week later at 170 g. wt. Given complete diet, litter born 21 days later, weaned 7 σ 1 g, av. wt. 33 g.

RIBOFLAVIN DEFICIENCY

Methods

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Cod-liver oil, 6 drops of a good sample per rat twice a week.	
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*Results**Effect on the vaginal contents*

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Rat	Wt. when first given deficient diet g.	First period of riboflavin deficiency days	Wt. when first given riboflavin g.	Dose of riboflavin μ g.	Wt. at end of dosing period g.	Second period of riboflavin deficiency days	Second daily dose of riboflavin (or milk)	Effect on vaginal contents	Reproduction
5038	60	43	(80)	0	(78)	23 anoestrus	20	3 cycles in 13, 15, 13 days	Mated at 140 g. wt. Given complete diet—no litter within 128 days
1828	07	43	(110)	0	(110)	23 anoestrus	20	3 cycles in 4, 7, 8 days	Mated at 164 g. wt. Given complete diet—no litter within 60 days
4845	60	13	110	1	130	23 anoestrus	20	3 cycles in 8, 13, 0 days	Mated at 160 g. wt. Litter born 23 days later, but dead in 3 days. Given 1 week's rest, remated at 105 g. Given complete diet—no litter within 110 days
5017	53	43	61	1	63	32 anoestrus	10 c.c. milk	3 cycles in 4, 7, 7 days	Mated at 120 g. wt., litter born 27 days later, but dead in 22 days. Remated at 185 g. Given 5 d 3 g, av. wt. 31.0 g.
5045	55	28	71	2.5	77	23 anoestrus	20,	1 cycle in 15 days, anoestrus for 20 days	Mated at 140 g. wt. Given complete diet, litter born 31 days later, weaned 5 d 3 g, av. wt. 39 g.
4830	50	43	60	2.5	90	23 anoestrus	20	3 cycles in 5, 6, 4 days	Mated at 127 g. wt. Given complete diet, litter born 24 days later, weaned 3 d 2 g, av. wt. 38.5 g.
5010	55	43	66	2.5	70	32 anoestrus	10 c.c. milk	3 cycles in 10, 14, 0 days	Mated at 103 g. wt. Given milk ad lib., litter born 24 days later, dead in 7 days. Given 1 week's rest, remated at 160 g. Given complete diet, litter born 28 days later, weaned 5 d 3 g, av. wt. 45 g.
4783	52	115	129	7.5	129	22 anoestrus	10 c.c. milk	3 cycles in 11, 12, 11 days	Mated at 185 g. wt. Given milk ad lib., litter born 37 days later, weaned 3 d 4 g, av. wt. 28 g.
1801	60	100	30	30	130	4 cycles in 7, 7, 15 days resp., then 20 days anoestrus	—	—	Mated at 140 g. wt. Given milk ad lib., born 25 days later, probably a litter. Given 1 week's rest, remated at 187 g. wt. Given complete diet, litter born 20 days later, weaned 1 d 7 g, av. wt. 38 g.
5018	47	100	30	135	135	3 cycles in 6, 14, 21 days resp., then 21 days anoestrus	—	—	Mated at 160 g. wt. Given milk ad lib., litter born 22 days later, weaned 2 d 6 g, av. wt. 20 g.
5175	37	90	30	113	113	24 anoestrus	10 c.c. milk	3 cycles in 8, 10, 10 days	Mated at 100 g. wt. Given milk ad lib., litter born 23 days later, dead in 3 days. Remated litter born at 170 g. wt. Given complete diet, litter born 21 days later, weaned 7 d 1 g, av. wt. 33 g.

the cycles of vaginal contents can be completely upset by a deficiency of riboflavin in the diet of the rat, and quickly restored to normal by giving riboflavin.

Effect on reproduction

Two rats which had been given only 1 μ g. riboflavin in the first part of the experiment (Lindholm's experiment) were mated after their cycles had been restored by daily doses of 20 μ g. riboflavin or 10 c.c. milk. Their weights at mating were 166 and 120 g. respectively. They produced live litters within 23 and 27 days respectively, but the litters died within 2 and 22 days. Five rats were given milk ad lib. after cycles were restored and all produced litters. Those which had early received 2.5, 30 and 30 μ g. respectively of riboflavin failed to wean their litters. Two which had early received 7.5 and 30 μ g. riboflavin weaned healthy litters. Then all the eleven rats were given the complete diet of our stock colony and all but three produced and weaned healthy litters within a very short time. Of the three which did not, two had received no dose of riboflavin in Lindholm's experiment, though the cycles of all three had been restored by giving 20 μ g. riboflavin daily. Hence a deficiency, total or almost total, of riboflavin in the diet resulted, in three out of four rats, in irreparable damage to some part of the reproduction process; a total deficiency for a shorter time, 28–52 days, followed by a partial deficiency for 21 days and again a total deficiency for 23–32 days has not resulted in irreparable damage, and normal reproduction has taken place when the rats were given a complete diet.

Only one report of a similar investigation has been found in the literature. That was by Engel & Phillips [1939], who found that a ration low in riboflavin for the chick had no adverse effect upon reproduction in the rat.

SUMMARY

The condition of anoestrus produced by withholding vitamin B₁ from the diet was corrected by administering vitamin B₁.

The disturbance in reproduction and lactation was not rectified by giving vitamin B₁, but when the stock diet of the colony was given, reproduction and lactation became normal, provided the period of deficiency had been not longer than 120 days.

A partial shortage of vitamin B₁ (and of an unidentified factor also) does not result in irreparable damage to the reproductive functions of the rat.

A deficiency of riboflavin in the diet of the rat also produces anoestrus, and normal cycles are restored by giving riboflavin.

This deficiency caused a disturbance in reproduction and lactation similar to that caused by a deficiency of vitamin B₁. Giving a complete diet corrected it in those rats which had suffered only a partial deficiency but not in those which had suffered a total or almost total deficiency for 68 days. Thus a total deficiency of riboflavin for about the first quarter of the rat's reproductive period caused irreparable damage. The damage caused by a partial deficiency was rectified.

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UREA FORMATION IN THE ISOLATED
PERFUSED LIVER OF THE RAT

BY Ó. A. TROWELL

*From the Department of Physiology, University of Edinburgh**(Received 22 September 1941)*

THIS paper is divided into two parts. The first describes a method of liver perfusion which is suitable for the quantitative study of liver metabolism. The second part reports an investigation of the mechanism of urea formation in the liver which has been carried out by this method.

LIVER PERFUSION

In the direct study of liver metabolism, perfusion of the isolated liver, introduced by Embden in 1901, has of recent years been largely superseded by the tissue-slice method of Warburg [1923]. Although some advances have been made in the technique of liver perfusion, there is still no method which permits of quantitatively accurate metabolic investigations at all comparable with those which may be achieved by the tissue-slice method. The failure of the perfusion method in this respect is due chiefly to the fact that it has been found impossible to maintain a constant rate of perfusion flow through the organ. It has been the general experience that in the perfused liver the rate of flow steadily declines and the liver swells up with oedema. The most recent work on liver perfusion, that of Bauer, Dale, Poulson & Richards [1932] and Chakravarti & Tripod [1940], has re-emphasized this conclusion. Furthermore, owing to the low perfusion pressure (portal) which has to be employed and the deformation which the liver, from its soft consistency, undergoes when removed from the body, the flow is often erratic and not evenly distributed throughout the liver. For metabolic investigations it is obviously essential that the rate of perfusion shall be adequate and shall remain reasonably constant throughout the experiment, for the rate of supply or removal of metabolites may be a limiting factor in the metabolic process. Further defects of the perfused liver are the loss of perfusion fluid which occurs from anastomotic vessels in the suspensory ligaments and bare area, and the difficulty of maintaining all parts of

the liver at a constant temperature. Also, in order to provide sufficient oxygen, it has been found necessary to perfuse with blood rather than saline and this makes chemical analysis and interpretation more difficult.

In the method to be described for perfusing the rat's liver it is claimed that these difficulties are largely overcome and that the method will yield results comparable in accuracy with those to be obtained by the tissue-slice method. The advantages of the rat are: that it is cheap, a standard strain of animals may be used, and that this animal has already been used for most of the liver-slice work. The perfusion method has certain advantages over the tissue-slice method which will be referred to later in connexion with urea formation.

PRELIMINARY OBSERVATIONS AND EXPERIMENTS

The rat's liver consists of seven semi-independent lobes, each of which receives a separate branch of the portal vein, hepatic artery and bile duct. The lobes are fused together at their bases forming a collar of hepatic tissue completely surrounding the inferior vena cava immediately below the diaphragm. Traced backwards, the vena cava runs for some distance within the caudate and right main lobes before emerging from the liver. During its intrahepatic course of about 1 cm. the vena cava receives the hepatic veins from the several lobes. Above the liver the vena cava receives the two phrenic veins and about 2 mm. below the liver it receives the right suprarenal vein; between these two points there are no tributaries other than the hepatic veins. This arrangement of the vena cava and its tributaries, which has been found consistently in a series of seventy animals, differs somewhat from the description given by Greene [1935]. There is usually no bare area in the rat, and so it is possible to pass a ligature round the vena cava between the liver and the diaphragm. Thin folds of peritoneum pass from the liver to the anterior abdominal wall, diaphragm, stomach and spleen, forming the falciform, left coronary, hepatogastric and gastrosplenic ligaments respectively.

The first attempts at perfusion were made as follows. A cannula, attached to a reservoir of warm oxygenated saline, was tied into the portal vein. The inferior vena cava was tied immediately below the liver. Another cannula, introduced into the thoracic part of the inferior vena cava, was passed down until its tip lay just below the diaphragm and secured by a ligature placed between the liver and diaphragm. After a preliminary perfusion to wash out the blood, the liver with cannulae was removed by cutting through the ligaments and diaphragm and set

up in a warm chamber. The effluent from the caval cannula was returned to the reservoir by a pump. Attempts to perfuse the liver in animals killed by stunning or asphyxia were always unsatisfactory, as persistent red patches showed that some parts of the liver remained unperfused. If, however, the above procedure was carried out under ether anaesthesia, then the liver became uniformly decolorized as soon as perfusion began and all the blood was quickly washed out. The liver has, therefore, always been removed under ether anaesthesia. In such a perfusion, using a pressure of 4-8 cm. H_2O , which approximates to the normal portal pressure in vivo, the flow was always erratic and much influenced by slight changes in the position of the liver and portal cannula; furthermore, it rapidly declined and the liver swelled up. A typical experiment is shown in Fig. 1 A. With the low perfusion pressure employed it seemed likely that the deformation of shape which the liver undergoes when removed from the body might be sufficient to kink some of the portal vein branches and so obstruct the flow. This difficulty was overcome by immersing the liver in a bath of saline so that the lobes floated out freely and assumed their natural positions. Under these conditions the flow was greater and fairly steady and could be kept constant for 2 hr. provided the perfusion pressure was raised a little towards the end. A typical experiment is shown in Fig. 1 B. In order to secure a constant flow for 2 hr. it was necessary to keep the perfusion pressure below 5 cm. H_2O . Raising it beyond this produced a greater initial flow, but the liver swelled greatly and the flow rapidly declined. Once the liver had swollen up it was impossible, ever again, to secure an adequate flow. Some leakage of fluid from the liver occurred in all these experiments.

Attention was now directed to the oxygen supply of the liver under these conditions. In previous work [Trowell, 1935] the oxygen consumption of rat liver slices was found to be 1.0-2.0 c.c./g./hr. Blalock & Mason [1936] found a liver oxygen consumption of 1.5-3.5 c.c./g./hr. in the conscious dog, and Barcroft & Shore [1912] 0.3-3.0 c.c./g./hr. in the anaesthetized cat. In the blood perfused liver, Jost [1931] found an oxygen consumption of 1.8-2.4 c.c./g./hr. and Lundsgaard, Nielsen & Ørskov [1936] 1.8 c.c./g./hr. in the cat. The oxygen requirement of the rat's liver may therefore be put at roughly 2 c.c./g./hr., and the average liver weight being 7 g., the total requirement is 14 c.c./hr. The solubility coefficient of oxygen in saline at 38° C. is 0.024, so that the maximum oxygen supply which can be provided by a flow of saline at 3 c.c./min. is 4.3 c.c./hr.; this is only about one-third of the requirement. Attempts to perfuse the liver with defibrinated blood, heparinized rat's blood and

haemoglobin-Ringer solutions were unsuccessful. The initial flow was low (less than 1 c.c./min.), and it rapidly declined to zero even if the perfusion pressure was considerably raised, so that any constant perfusion was quite impossible. Saline has been used in all further work, and the

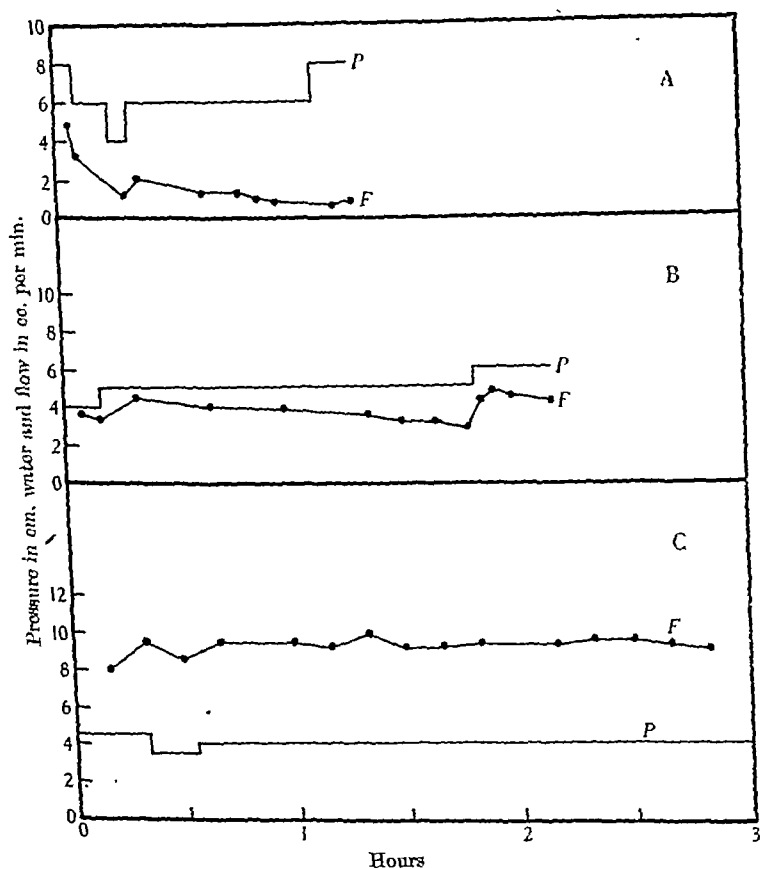


Fig. 1. Perfusion pressure (P) and rate of flow (F) under varying conditions. A. Liver exposed to air, forward perfusion. B. Liver immersed in saline, forward perfusion. C. Liver immersed in saline, backward perfusion.

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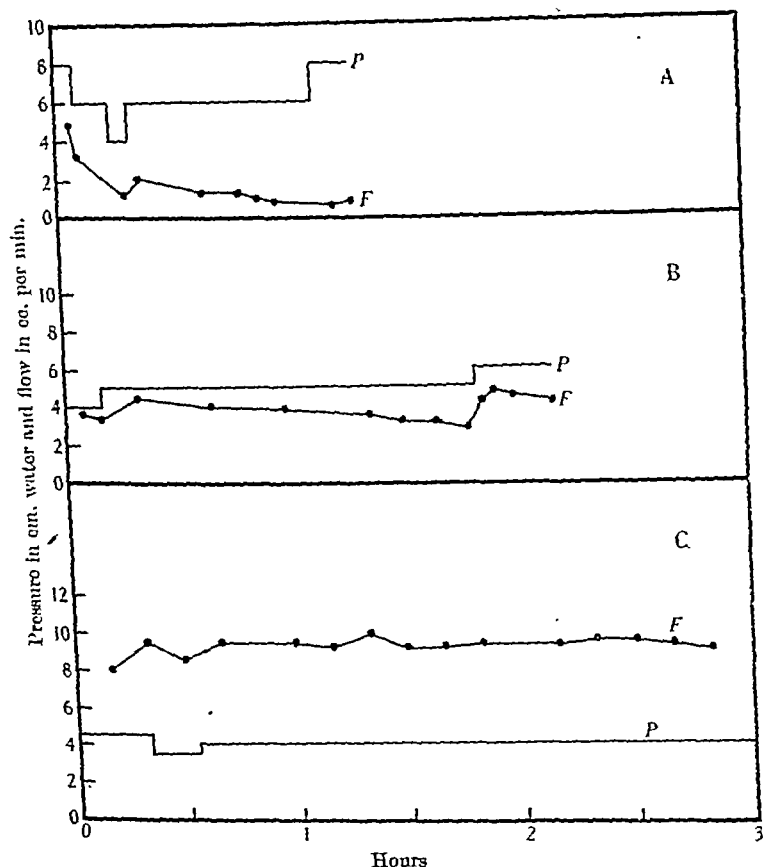


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in the normal forward direction. Furthermore, the flow was more constant than with forward perfusion, as may be seen in Fig. 1C. The explanation of this curious fact has not been discovered, but it is a constant phenomenon and the success of the method is chiefly due to it. Now, with a perfusion pressure of 4–6 cm. H_2O and a flow of 8–10 c.c./min. the oxygen supply is 11.5–14.5 c.c./hr. This oxygen supply would seem to be fairly adequate, but it is probably not always optimal. The main features of the method are, then, that the liver is removed under anaesthesia, is immersed in saline and is perfused backwards.

The perfusion apparatus

The apparatus is shown in Fig. 2. It consists essentially of two glass vessels and a pump. One vessel, *L*, is the liver bath and the other, *O*, serves both as a perfusion reservoir and an oxygenator. Saline from *O* flows into the liver by gravity via a cannula in the inferior vena cava. The saline, after perfusing the liver, escapes from the cut portal vein directly into the liver bath, from which it is returned to *O* by a roller pump *P*. The saline of the liver bath is thus incorporated in the general perfusion circuit, there is no portal vein cannula and the liver floats in its own perfusion fluid. When a cannula is tied into the portal vein the main lymphatic vessels leaving the liver are automatically tied off and this contributes to the liver swelling. The absence of a portal vein cannula, therefore, means that there is no lymphatic obstruction and the 'lymph' drains into the liver bath. The oxygenator vessel is shaken to and fro after the fashion of a Warburg manometer in order to ensure equilibration with the gas mixture.

The whole of the perfusion circuit, with the exception of the pump, is immersed in a glass water bath, *W*, maintained at 38° C. by an electric immersion heater, thermoregulator and stirrer. The oxygenator vessel is a small cylindrical bottle such as is commonly used for perfusing the frog's blood vessels. This bottle is held horizontally in a retort-stand clamp which is pivoted at *B* on to a horizontal arm carried by a vertical stand *S*. The far end of the clamp is attached to a crank, *C*, driven by a motor so that the bottle is shaken from side to side through an arc of about 4 cm. The rate of shaking is fairly critical; at the proper rate the fluid is swished evenly round the walls without any splashing or frothing. With the particular vessel used here the rate had to be kept between the limits 170–180 oscillations/min. Owing to the shaking of the oxygenator the perfusion pressure is slightly pulsatile; this is probably an advantage. It may be noted here that bubbling oxygen through the perfusion fluid

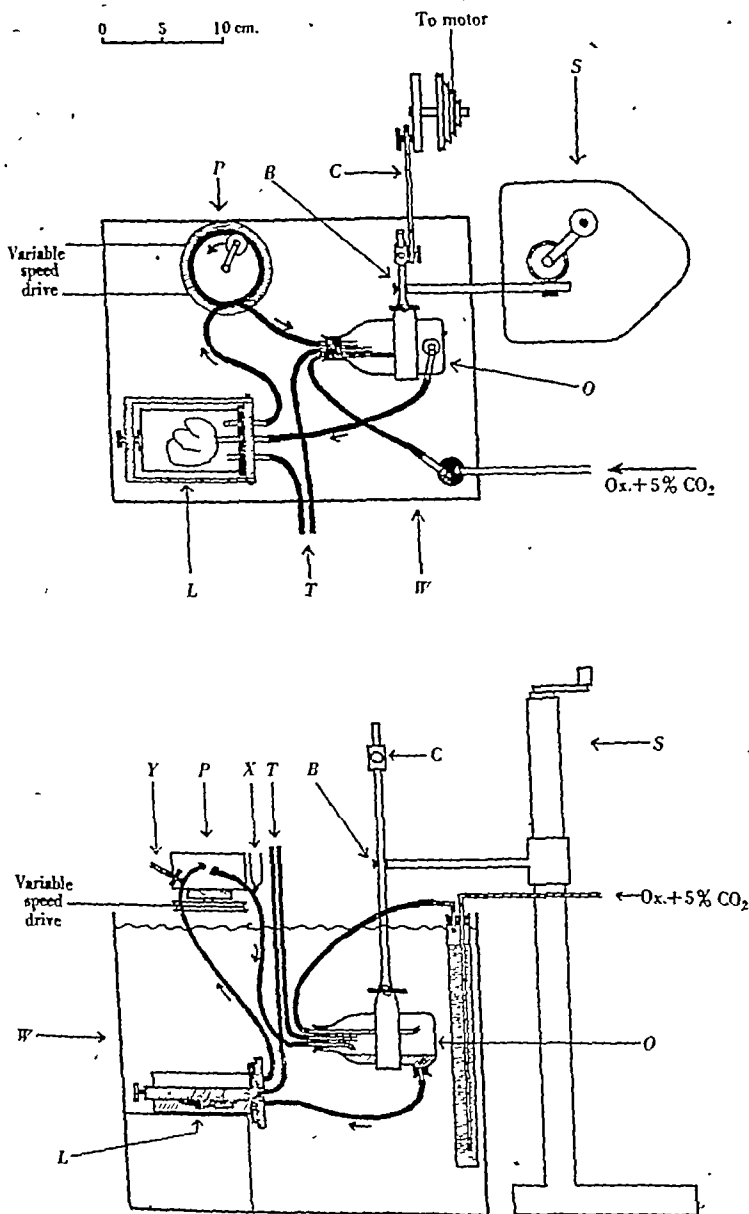


Fig. 2. Scale drawing of the perfusion apparatus. Plan above and elevation below. Heating arrangements and electric motors not shown.

is unsatisfactory owing to the frothing produced. The stand, *S*, is provided with a screw handle by means of which the horizontal arm can be racked up or down, and in this way the perfusion pressure can be varied at will. As the crank, *C*, is fairly long this adjustment does not upset the shaking arrangements. The pump, *P*, consists of a loop of

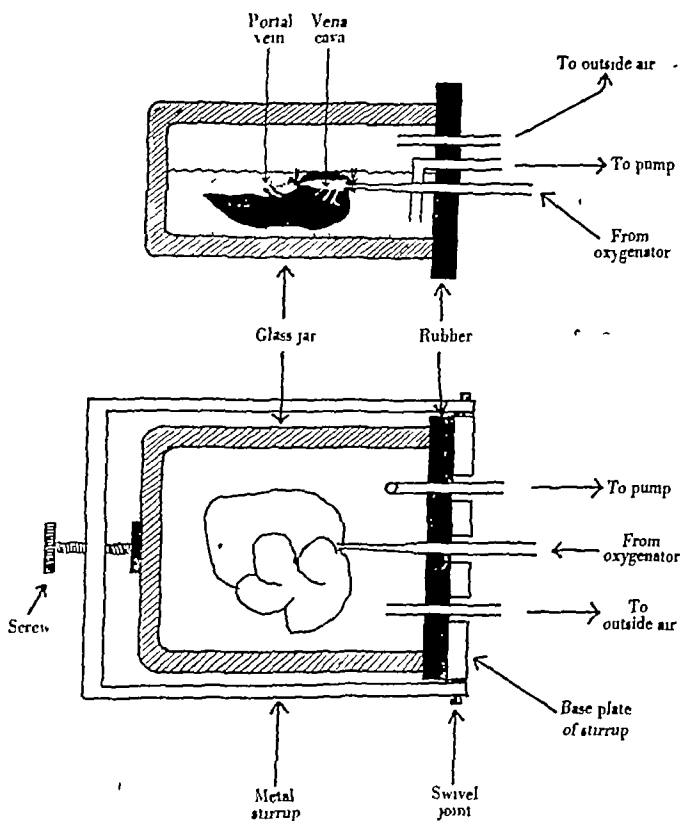


Fig. 3. The liver bath. Vertical section above and horizontal section below.

rubber tubing running round the inside of a metal cylinder. A rotating arm within the cylinder carries a small metal roller which compresses the tubing against the cylinder and so drives the fluid along the tube. The output of the pump when fitted with $\frac{3}{32}$ in. diameter tubing is 0.8 c.c. per revolution. The pump is driven from a constant-speed electric motor via a friction-plate type of variable-speed gear (Palmer). The output per minute of the pump can thus be very accurately controlled and will remain constant at any one setting. During the perfusion the

speed of the pump must be carefully adjusted so that its output is exactly equal to the rate of flow through the liver, under which conditions the fluid level in *L* will remain steady. Any change in the rate of flow through the liver will be reflected in a slow change of the level in *L*, and this has to be compensated for by altering either the speed of the pump or the perfusion pressure. In practice, once the initial adjustment has been made and the level stabilized, further adjustment is usually only necessary at half-hourly or longer intervals. The variable speed gear is calibrated, so the output of the pump is always known, and, so long as the level in *L* is steady, this may be taken as a measure of the rate of flow through the liver.

The saline used is that introduced by Krebs & Henseleit [1932]. This saline reproduces the inorganic composition of rat plasma very much more closely than the usual Ringer-Locke or Tyrode solutions, it is buffered with bicarbonate and when equilibrated with 5% CO₂ has a pH of 7.4. The gas mixture passed through the oxygenator is 95% O₂ and 5% CO₂, previously warmed and saturated by bubbling through a vessel of water held in the main water bath.

The liver bath (Fig. 3) consists of a heavy glass museum jar measuring, internally, 3 × 5.5 × 7 cm. The jar is laid flat on its side and the open end is closed by a sheet of $\frac{1}{4}$ in. thick rubber held firmly against the ground edges of the jar by a heavy metal stirrup which can be screwed up tight. The three glass tubes which enter the bath pass through tightly fitting holes in the rubber sheet, and the base-plate of the stirrup has three larger holes to give clearance to these tubes. The cannula is made from 2.5 mm. bore glass tubing, the final 2 cm. being drawn down to 1 mm. bore. The tip is cut squarely and flanged; the external diameter of the flange is 2 mm. The tubes, *T* (Fig. 2), are open to the air and maintain atmospheric pressure in the two vessels.

It is necessary to prove that all the fluid circulating is, in fact, passing through the liver and that none is escaping directly into the liver bath from some leak in the vena cava. A simple test was devised based on the fact, discovered in earlier experiments, that when a 1 in 5000 solution of methylene blue is perfused through the liver it emerges completely decolorized. This is not due to reduction to the leuco form, for reoxygenation fails to restore any colour; apparently the liver cells remove all the dye from the fluid during a single passage through the liver. The absorption of methylene blue is completely inhibited by traces of cyanide. Now when methylene blue is introduced into the oxygenator so that dark blue fluid enters the liver, no blue whatever comes through into

the fluid of the liver bath. This proves that all the fluid passes through the liver sinusoids and comes into contact with active liver cells. This test may be applied at any time during the course of a perfusion, and repeated tests have shown that leakage never occurs. For the purpose of metabolic experiments this perfusion system has the advantage that even if a small leak were to occur the fluid would not be lost from the circuit, and, further, any fluid escaping from the bile duct, hepatic artery or lymphatics is retained in the circuit.

Method of setting up the perfusion

A cylinder containing 130 c.c. of the saline is stood in the warm water bath and the gas mixture set bubbling through it. The rat is anaesthetized, the whole anterior wall of the thorax and abdomen is removed, the falciform ligament being divided and the anterior part of the diaphragm cut away to within a few millimetres of the caval opening. The phrenic nerves are divided to immobilize the diaphragm, and a femoral vein is opened to relieve the asphyxial congestion of the vena cava and liver. A No. 0 silk ligature is now passed round the inferior vena cava between the liver and the diaphragm and the first loop of a knot loosely tied. This is a somewhat difficult procedure, as the liver and vena cava are easily damaged; the best instrument for the purpose is the eye end of a curved surgical needle (size 3 eye needle), the point of the needle being inserted into a wooden holder. The threaded needle is passed from right to left under the vena cava and brought up between the cava and the oesophagus, where it is sometimes necessary to pierce a few strands of connective tissue (a rudimentary bare area). In a few animals it has been found impossible to pass this ligature owing to the presence of a bare area between the vena cava and oesophagus; such animals have to be discarded. The left phrenic vein runs down alongside the vena cava and enters that vessel several millimetres below the level of the diaphragm and almost within the liver substance. The left phrenic vein must therefore be included within the vena cava ligature so that, when the cannula is eventually tied in, this vein will automatically be tied off. A ligature is now passed round the vena cava immediately below the liver and the first loop of a knot loosely tied. The cannula is introduced through an incision in the right auricle and passed down the inferior vena cava until its tip lies just below the diaphragm, where it is tied in place. The flange of the cannula is pulled back against the ligature and kept clear of the liver so that there is no risk of obstructing the mouths of the hepatic veins. The cannula is now filled up with saline

and connected to a small perfusion bottle containing about 40 c.c. of the warm equilibrated saline, so placed that the perfusion pressure will be about 4 cm. H_2O . The vena cava is now opened somewhere below the lower ligature, whereupon saline escapes and flushes out the blood from the cannula and hepatic part of the vena cava. The lower ligature is tied and the portal vein cut off as close to the liver as possible. The saline will now perfuse the liver and escape from the portal vein stump. It may be necessary to raise the perfusion pressure to 8 cm. H_2O at first in order to wash out the blood, but thereafter it is kept at 4 cm. After about 5 min. perfusion the effluent is free from blood, the cannula is now disconnected from the bottle and, after cutting through the remaining ligaments and the diaphragm, the cannula with liver attached is lifted out. 20 c.c. of saline are now run into the oxygenator and 60 c.c. are placed in the jar of the liver bath which is stood upright on the bench. The cannula is pushed through the hole in the rubber sheet and the liver lowered into the jar. The stirrup is placed in position over the rubber sheet and screwed up tight. If necessary, the cannula is rotated so that the liver will ultimately lie posterior surface uppermost. The liver bath may now be laid horizontally, the rubber tubes are connected to it and it is lowered into the water-bath. The pump and shaker are now started and the perfusion pressure and pump speed appropriately adjusted. The time taken from the start of the operation until the perfusion is fully set up is 15-20 min., and the circulation through the liver is arrested for about 5 min. in all.

Behaviour of the perfused liver

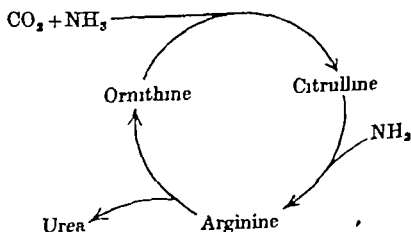
In a series of forty-seven perfusions, rats varying in age from 3 months to 3 years have been successfully used. The best results were obtained with animals 6-9 months old weighing 170-230 g. In such animals a perfusion flow of 8-10 c.c./min. can always be maintained for 2-4 hr. The liver volume increases by about 20% during the course of the perfusion. The perfusion fluid is generally slightly opalescent owing to the presence of a few red cells. After 1-2 hr. perfusion these red cells suddenly haemolyse and the fluid is then quite clear and faintly yellow. A certain amount of protein slowly accumulates in the perfusion fluid, and after 2 hr. or more some of it may separate out in the form of a coagulum. This coagulum only forms in the oxygenator, and it is probably due to mechanical denaturation brought about by the shaking. The coagulum collects into a small lump or thread which floats on the surface and shows no inclination to pass down into the cannula. In four experiments the liver was examined histologically after 3 hr. perfusion. The central

veins and sinusoids were widely dilated and the portal tracts showed some oedema. The Kupffer cells were rounded up. The liver cells were slightly swollen and the cell walls were unusually distinct. The cytoplasm of the liver cells was somewhat oedematous, staining more palely than usual, but the nuclei were normal. The histological appearances were the same in all parts of the liver, indicating that the perfusion had been fairly uniform.

UREA FORMATION

It is known that the liver can form urea from ammonia plus CO_2 , from arginine, from glutamine and asparagine plus ammonia [Leuthardt, 1938] and possibly from histidine and proline [Borsook & Jeffreys, 1935]. It is still believed that in normal life most of the urea is formed from ammonia and CO_2 , but the evidence will not be discussed here. This paper is only concerned with the chemical mechanism involved in the synthesis of urea from ammonia and CO_2 . In respect of this various theories have been advanced. The simple view that ammonium carbonate is first formed and that this loses 2 mol. of water to form urea was generally accepted until quite recently. There is no experimental evidence either for or against this theory; its recent decline in popularity has been due to the suggestion of rival theories. The theory of Werner [1923] that urea has a cyclic formula and that cyanic acid is the intermediary has been disproved by Sumner and his colleagues [1931].

Modern work on urea formation dates from Krebs & Henseleit [1932], who, working with rat-liver slices, made the fundamental discovery that ornithine catalyses the formation of urea from ammonia and CO_2 . It was known that the liver contains an enzyme, arginase, which breaks arginine to urea and ornithine; so they supposed that ornithine combines with ammonia and CO_2 to form arginine which would then break down to urea and ornithine and the cycle would go on indefinitely. They also supposed that citrulline was an intermediary between ornithine and arginine. The complete scheme, the 'ornithine cycle', may be depicted thus:

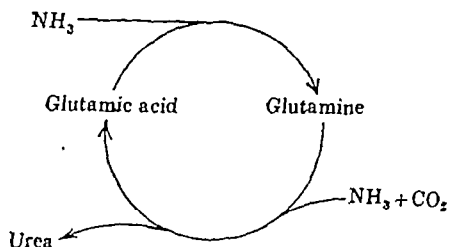


The essential 'catalyst' which is ornithine, citrulline or arginine, according to the stage of the cycle, is not used up, so the addition of a small amount of any one of these substances should bring about a permanent increase in the rate of urea formation from ammonia. The evidence put forward by Krebs & Henseleit was as follows. They showed that ornithine in concentrations of 10 mg. % or less markedly increased the rate of urea formation from added ammonia. Up to 30 mol. of extra urea were formed per molecule of ornithine added and ornithine in the absence of ammonia formed no urea, so the effect was truly catalytic. Arginine, since it is broken down to ornithine by arginase, naturally exhibited a similar catalytic action. The addition of citrulline was shown to increase urea formation, but since in all the experiments a concentration of 200 mg. % was used the effect was not shown to be catalytic. The rest of the evidence concerned the ratio of molecules of ammonia disappearing to molecules of urea formed. With ornithine the ratio should be 2.0 and the figure found was 1.81; with citrulline the ratio should be 1.0 and the figure found was 1.38. The evidence for this theory is incomplete in two respects: first, there is no evidence that ornithine is ever converted into arginine and, secondly, a catalytic effect of citrulline has not been demonstrated.

The experimental findings of Krebs & Henseleit have been confirmed by other workers, but further evidence in support of the theory has not been forthcoming. The effect of ornithine on liver slices was confirmed by Gorter [1937] and by Manderschied [1933]. Bach [1939] confirmed the fact that 200 mg. % citrulline increases urea formation in the presence of ammonia. He also found that citrulline could give rise to urea in the absence of ammonia and without being converted to arginine, and suggested that citrulline reacted with α -keto-glutaric acid to form glutamic acid and urea. Ikeda [1938], in the perfused dog's liver, found that citrulline increased the yield of urea from added ammonia, and also that citrulline alone gave rise to some urea. The effect of ornithine has been denied by London & Alexandry [1937]. These workers removed blood samples from the hepatic and portal veins of the conscious dog and found that injection of arginine increased the urea content of hepatic vein blood, but that ornithine had no effect. Gorter [1937] found that if rats were kept for several days on a protein-free diet then ornithine had no effect on liver slices although the rate of urea formation from ammonia was normal.

Bach [1939] has put forward another theory of urea formation. Working with rat-liver slices he found that both glutamic acid and

glutamine increased the rate of urea formation from ammonia and suggested the following scheme of an 'amide nitrogen cycle':



He also suggested that ornithine and citrulline might be oxidized by α -keto acids to glutamic acid or glutamine, and that the results of Krebs & Henseleit might thus be interpreted in terms of the amide nitrogen cycle. This, however, seems unlikely, as Bach used concentrations of 100 mg. % to demonstrate effects with glutamine, whereas Krebs & Henseleit had demonstrated an effect with ornithine at 1 mg. %.

The present position is that several mechanisms of urea formation have been suggested; no one of them has been proved ever to occur and the normal process of urea formation from ammonia is still obscure. The object of the present work was to investigate the effects of ornithine, arginine, citrulline, glutamine and glutamic acid on the rate of urea formation from ammonia in the perfused liver, using low concentrations which might be supposed to be within the physiological range (10–20 mg. %). In this way it was hoped to obtain more satisfactory evidence with regard to the ornithine cycle and the amide nitrogen cycle. For such an investigation the perfusion method has some advantages over the tissue-slice method. With the tissue-slice method, apart from the oxygen consumption, it is only possible to determine the total amount of metabolite formed or removed at the end of the experiment. In order to discover the effect of any experimental procedure on the rate of a metabolic process it is necessary to perform parallel experiments, and, owing to the individual variation of the slices, a large number of experiments has to be performed in order to establish a statistically significant result. In the perfusion method, on the other hand, by analysis of samples of perfusion fluid removed at intervals, the rate of a metabolic process can be followed throughout the experiment and quite small experimentally produced changes of rate can be convincingly demonstrated.

METHOD

The liver was perfused with saline as described, 1.0 c.c. samples of perfusion fluid were taken at 15 min. intervals, and the urea content estimated by the manometric urease method of Krebs & Henseleit [1932]. The samples were removed from a short wide tube of 2 c.c. capacity (Fig. 2, X) which is filled by the pump when the rubber tube beyond is clipped. Substances to be added during the experiment were made up in concentrations such that the volume to be added was about 1.0 c.c. This was run down into the liver-bath through a side tube (Fig. 2, Y), the pump being stopped. Thus it was ensured that the added substance was fully diluted before perfusing the liver.

In each experiment urea concentration was plotted against time, and from the average slope or slopes of the curve Q urea values were calculated (Q urea = urea as cu. mm. CO_2 equivalent/mg. dry wt./hr.). For this calculation it is necessary to know the total volume of liver plus perfusion fluid. This can be measured at the end of the experiment. During the experiment, however, the total volume decreases by about 10% owing to the removal of samples, and the actual volume present at any time must be calculated. The total volume at the end of the first hour has been taken as the 'mean total volume' for the purpose of calculating Q urea. Owing to the reduction in total volume during the experiment the urea concentration/time curve is not a strictly accurate representation of the rate of urea formation. It is possible to correct for this by calculation, but the correction turns out to be very small and such correction has not been made in the results reported here. The significance of the results is not thereby affected. At the end of each experiment the liver was dried at 100°C . to constant weight.

The rats used were 4-7 months old taken from an albino strain bred in the laboratory and fed on a standard diet. The chemicals used were ammonium chloride (B.D.H.) and the l (+) forms of ornithine dihydrochloride, arginine carbonate, citrulline, glutamic acid and glutamine. The amino acids were those of Roche Products Ltd., and the glutamine was kindly supplied by Dr R. L. M. Synge. The solutions were adjusted to pH 7.4 before addition.

RESULTS

Ammonia

With saline alone there was practically no urea formation. In five experiments there was a slight formation and in eight none at all. When ammonia was added (as the chloride) urea formation occurred at a rate

which remained fairly steady for 4 hr. (Fig. 4). Q urea values in the presence of 10–14.5 mg. % ammonia were measured in the course of many experiments and ranged from 0.3 to 1.31 (Table 2). Krebs & Henseleit [1932], using liver slices and a similar concentration of ammonia, found considerably higher values (roughly 1.0–5.0), but Bach [1939] found practically no urea formation from ammonia alone. It is of interest here to mention three experiments in which the average Q urea of the rat's liver during normal life was measured by correlating the urinary urea output over 24 hr. with the dry weight of the liver. The figures found were 0.9, 1.28, 1.7. The rate of urea formation in the perfused liver therefore is somewhat less than the normal rate *in vivo*. Krebs & Henseleit found that urea formation did not occur in the absence of

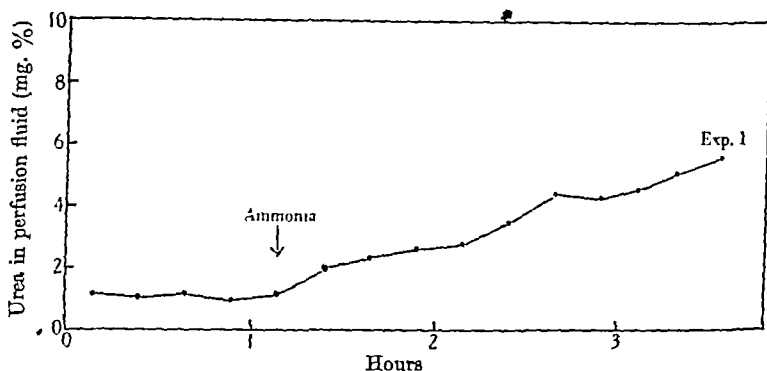


Fig. 4. Urea formation from ammonia.

oxygen, and that the addition of respiratory substrates such as fructose, lactate and pyruvate increased urea formation from ammonia. Salaskin & Kriwsky [1931], in the saline perfused dog's liver, found no urea formation from ammonia, but urea formation occurred when red cells or haemoglobin were added to the saline. The rather low Q urea values found here might therefore be attributed to a deficient oxygen supply. It has already been pointed out that with this perfusion method the oxygen supply may not be optimal. Nevertheless, it seems unlikely that oxygen was a limiting factor in these experiments, for in later experiments with ornithine much higher Q urea values were obtained. The addition of fructose or glucose (200 mg. %) was found to have no effect on the rate of urea formation from ammonia.

In the experiments now to be described, except where otherwise stated, the concentration of ammonia has been 10–14.5 mg. %, and the concentration of the amino acid 10–20 mg. %. Details are given in Table 2.

Ornithine

The catalytic effect of ornithine was fully confirmed. Three experiments are shown in Fig. 5, in which ammonia was present from the start and ornithine added at the point indicated. In Exp. 6 the addition of 9.7 mg. ornithine led to an 'extra' urea formation which amounted to

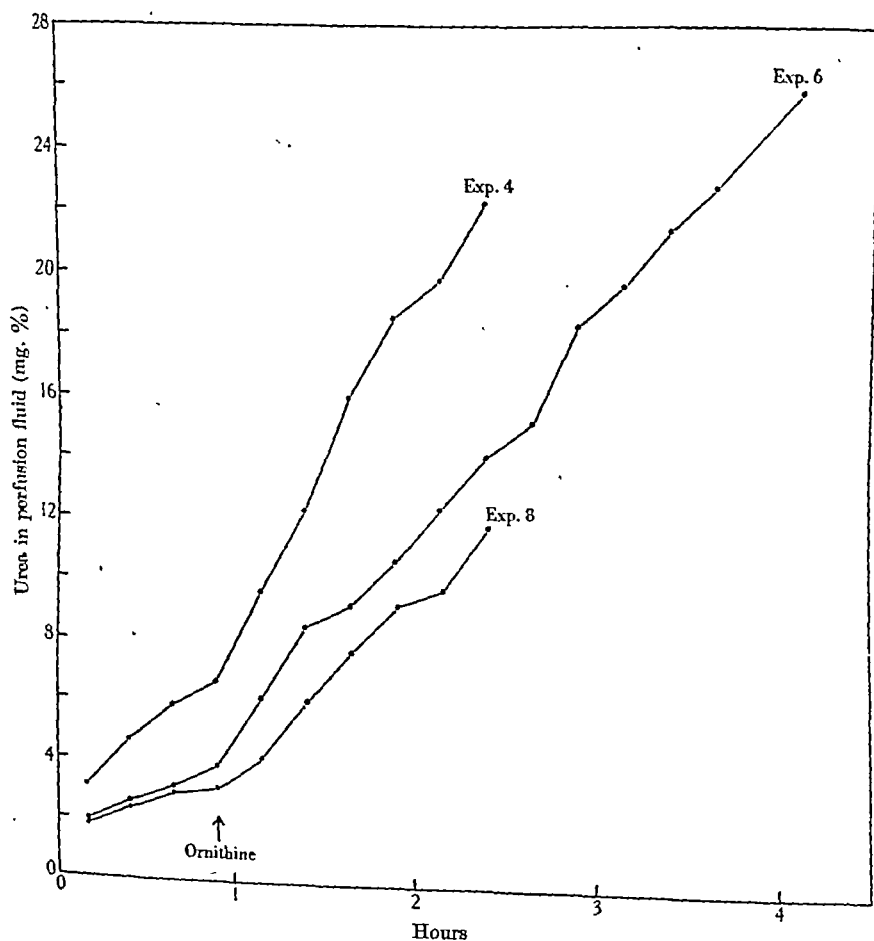


Fig. 5. The effect of ornithine in the presence of ammonia.

13 mg. by the end of the experiment. This is a ratio of 3.5 mol. urea to 1 mol. ornithine, so the effect is truly catalytic. In Exp. 9 (Fig. 6) ornithine alone was present at the start and the urea formation was almost nil; ammonia was added after 1 hr. and the Q urea at once rose

to 2.28. The direct conversion of ornithine to urea is therefore ruled out.

Making use of the accelerating effect of ornithine it was possible to measure the yield of urea from a given amount of ammonia added. Two such experiments are shown in Fig. 7. Ornithine was present from the

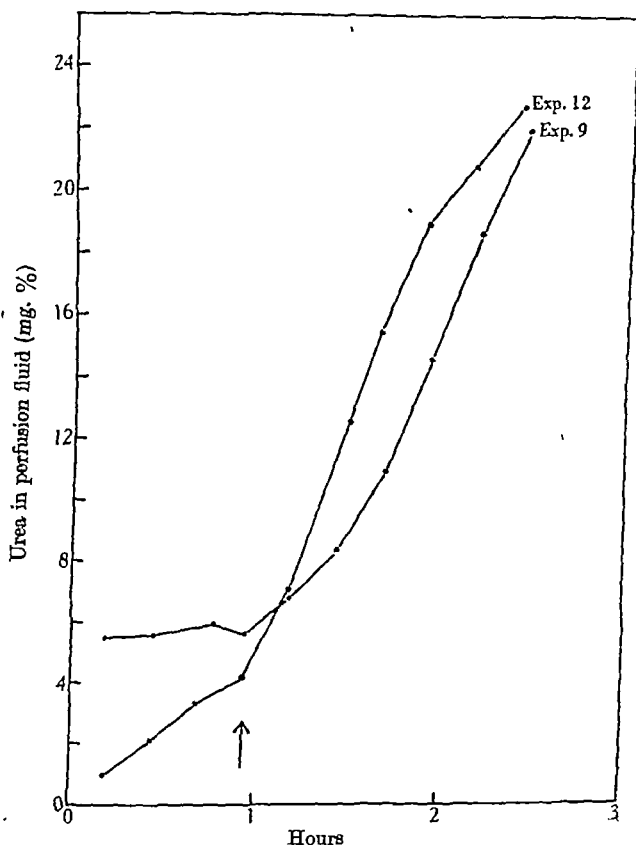


Fig. 6. Exp. 9. Ornithine present throughout, ammonia added at arrow.

Exp. 12. Ammonia present throughout, arginine added at arrow.

start, and after the initial rate of urea formation had been measured (zero in these two cases) a small measured quantity of ammonia was added and the experiment continued until the urea formation returned to its former level. The extra urea formed was calculated from the increase in concentration, knowing the total volume of liver plus perfusion fluid (Table 1). The percentage of the ammonia recovered as urea was rather low, indicating that ammonia is used up in other reactions. The fact that

with the lower concentration of ammonia proportionally less was converted into urea possibly indicates that these other reactions have the first call on the ammonia. An alternative explanation would be that there is a limiting concentration of ammonia below which urea formation does not occur.

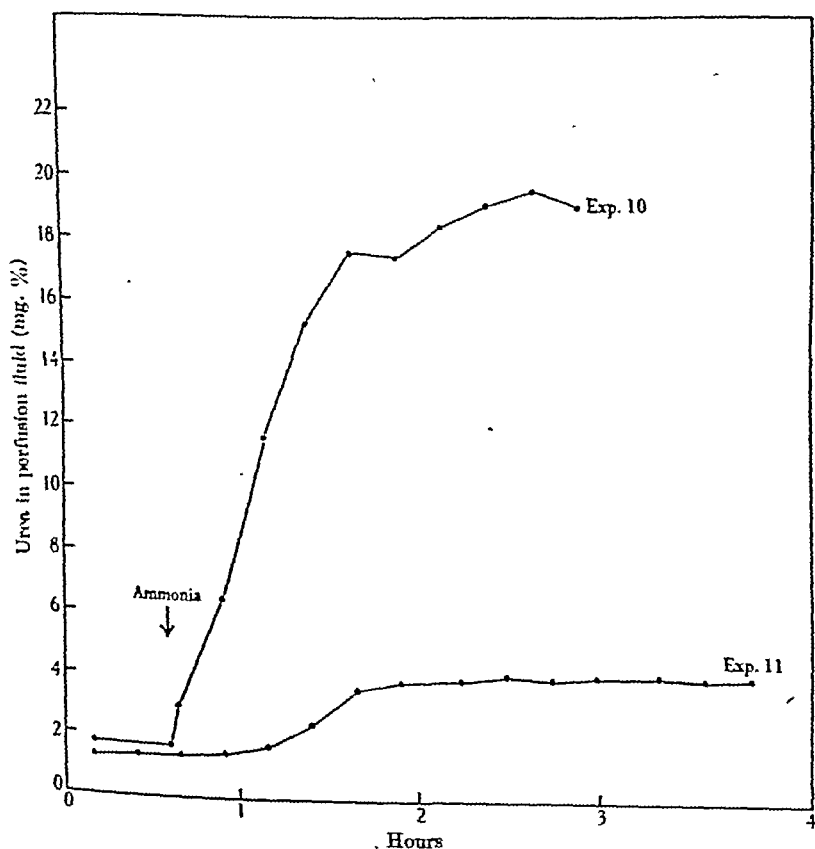


Fig. 7. Quantitative yield of urea from added ammonia. Ornithine present throughout, 10.5 mg. ammonia added in Exp. 10 and 3.2 mg. in Exp. 11.

TABLE 1. Quantitative yield of urea from (i) ammonia; and (ii) arginine

Exp. no.	Substrate added mg.	Urea formed mg.	Percentage of substrate recovered as urea
10	Ammonia, 10.5	14.2	76
11	Ammonia, 3.2	2.5	45
13	Arginine, 16.4	5.5	97
14	Arginine, 16.4	6.0	105
15	Arginine, 16.4	6.8	120

Arginine

In the presence of ammonia, arginine, as was expected, had the same sort of effect as ornithine (Exp. 12, Fig. 6). Now arginine, since it breaks down to urea and ornithine, should give a urea formation even in the absence of ammonia. Furthermore, since this urea formation from arginine is one of the steps in the ornithine cycle it should proceed at a rate which is at least equal to the rate of urea formation from ammonia

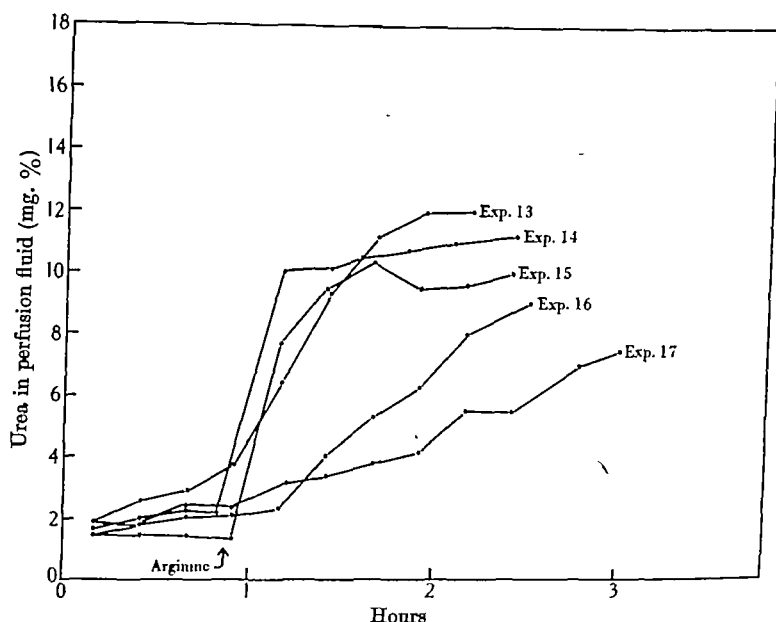


Fig. 8. Urea formation from arginine, in the absence of ammonia.

in the presence of a similar concentration of ornithine. In other words; we are here investigating the rate of one of the component reactions of the ornithine cycle, and, if the theory is correct the rate of any one component reaction cannot be less than the rate of the cycle itself. The rate of urea formation from arginine alone was investigated in eight experiments, five of which are shown in Fig. 8. It was found that the rate was very variable in different livers. In five out of the eight experiments the rate of urea formation was definitely less than that obtained in any of the experiments with ornithine and ammonia (see Fig. 13). This result makes it difficult to believe that in the ornithine experiments *all* the urea was formed by means of the ornithine cycle.

In some of these experiments ammonia was added some time after the arginine, with the following results. In Exps. 13 and 15 (Fig. 9) ammonia was added after all the arginine had been converted to urea

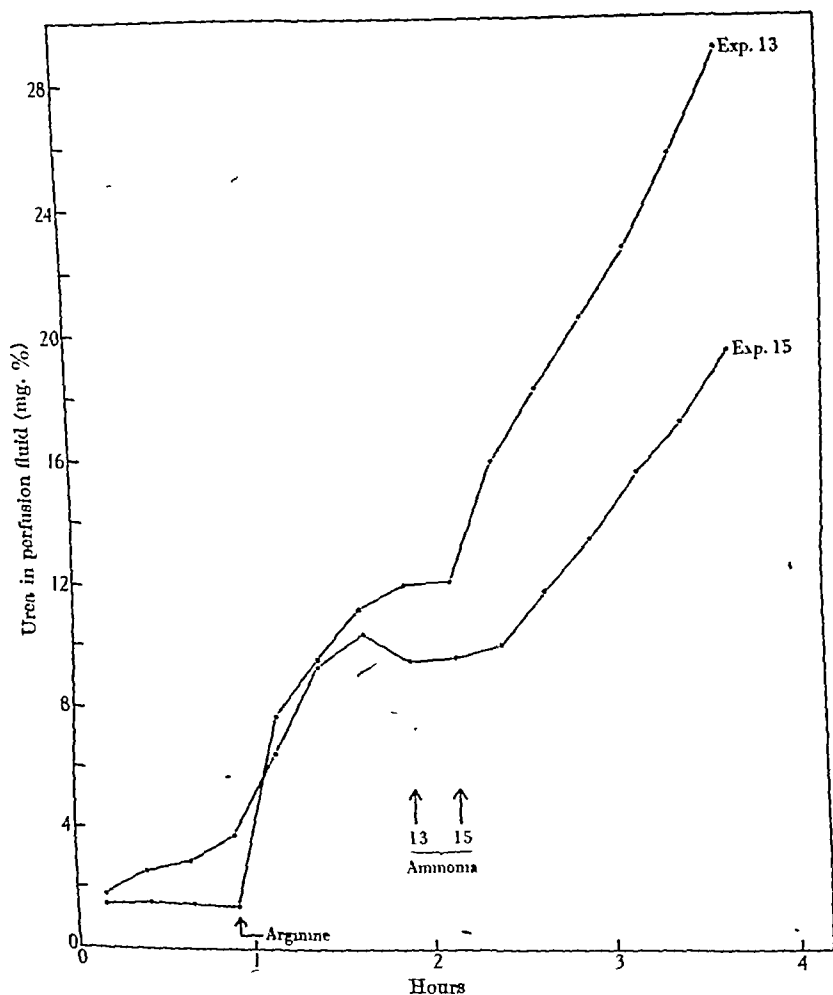


Fig. 9. The effect of adding ammonia after all the arginine had been converted to urea and ornithine.

and ornithine; the rate of urea formation was now about the same as had previously been obtained with ornithine and ammonia and slightly less than the rate of formation from arginine alone. This fits in with the ornithine cycle theory. It is interesting to note that such high and constant rates of urea formation can be obtained after 3-4 hr. perfusion.

On the other hand, in experiments in which the rate of urea formation from arginine was slow it was found that the addition of ammonia definitely increased the rate of urea formation above that obtained with the arginine alone (Fig. 10). Now in terms of the ornithine cycle, the only effect of adding ammonia at this stage would be to resynthesize the very small amount of arginine which had already broken down to ornithine, and restoration of the concentration of arginine to its original level would not increase the rate of urea formation, for, as the curves show,

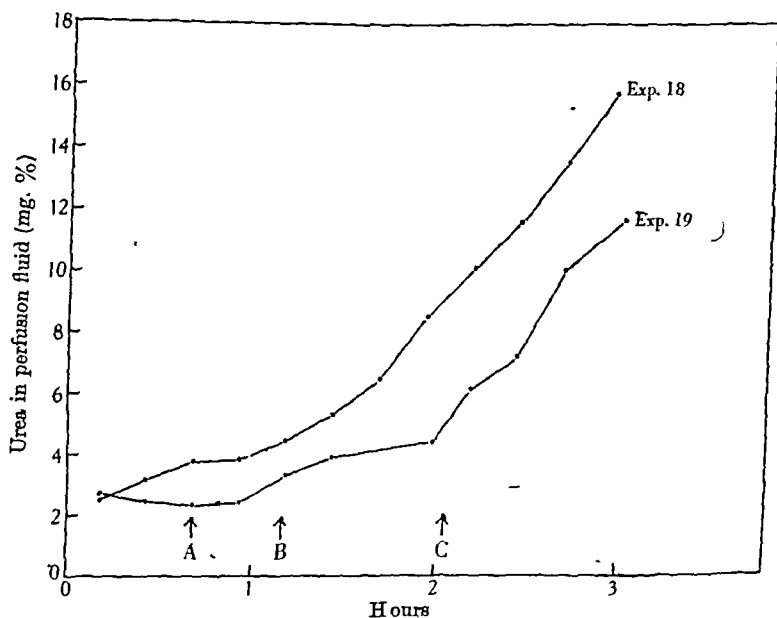


Fig. 10. Exp. 18. Arginine present throughout, ammonia added at B.
Exp. 19. Arginine added at A, ammonia added at C.

the rate had not fallen off anyway. It is also noteworthy, comparing Figs. 9 and 10, that in spite of great differences in the rate of arginine breakdown the rate of urea formation from ammonia was about the same. Taken as a whole, these experiments show that in some livers the rate of arginine breakdown is slow, but that in such livers the rate of urea formation from ammonia is not correspondingly limited. This leads to the conclusion that some urea at any rate is being formed by a mechanism other than the ornithine cycle.

These variable results obtained with arginine were not due to arginase contamination of the stock arginine solutions; strict precautions were

taken to avoid this, and the results obtained bore no relation to the age of the solutions. The experiments were performed in the following order, 19, 17, 18, 15, 16, 14, 13, using the same stock solution. The variation could not be accounted for by differences in the arginase content of the livers. In seven rats from the same strain the arginase content of the liver was measured by the method of Baldwin [1935]. Expressed in the units defined by Baldwin the results showed a range of 340-555. It is unlikely that variations in the perfusion conditions were responsible, for with other substances, e.g. ornithine, the results have been fairly constant.

In Exps. 13, 14 and 15 (Fig. 8) the quantitative yield of urea from arginine was measured. The results are given in Table 1 and are of interest in that they afford a useful check on the accuracy of this perfusion method for quantitative work.

Citrulline

The effect of citrulline in the presence of ammonia was investigated in six experiments. The concentration of citrulline was 10-20 mg. %, the same as had been used in the case of ornithine and arginine. In one experiment (23) a small increase in urea formation occurred, in the other five there was no effect at all. The one positive and two of the negative experiments are shown in Fig. 11. As has been mentioned, other workers have always used higher concentrations. Fig. 12 shows three experiments in which a concentration of 105-115 mg. % was used. At this concentration citrulline gave rise to a small urea formation even in the absence of ammonia, while in the presence of ammonia the urea formation was quite large. A further important point was demonstrated, and one which would not be detectable in liver-slice experiments, namely, that the effect of citrulline is only transitory, for within an hour or so the urea formation has returned to its former level. The effect of citrulline is clearly not catalytic. The effect was not due to the presence of urea as an impurity in the citrulline, for in that case the urea would have appeared much more quickly; neither was it due to the presence of ornithine or arginine as impurities, for in that case the effect would have been catalytic. These experiments also prove that citrulline is not converted either into ornithine or arginine, for, had it been, a persistent increase in the rate of urea formation would have been observed. About 7 mg. of urea were formed as the result of adding 100 mg. of citrulline (in the presence of ammonia). This corresponds to 1 mol. of urea per 7 mol. of citrulline, a result which does not admit of any simple chemical

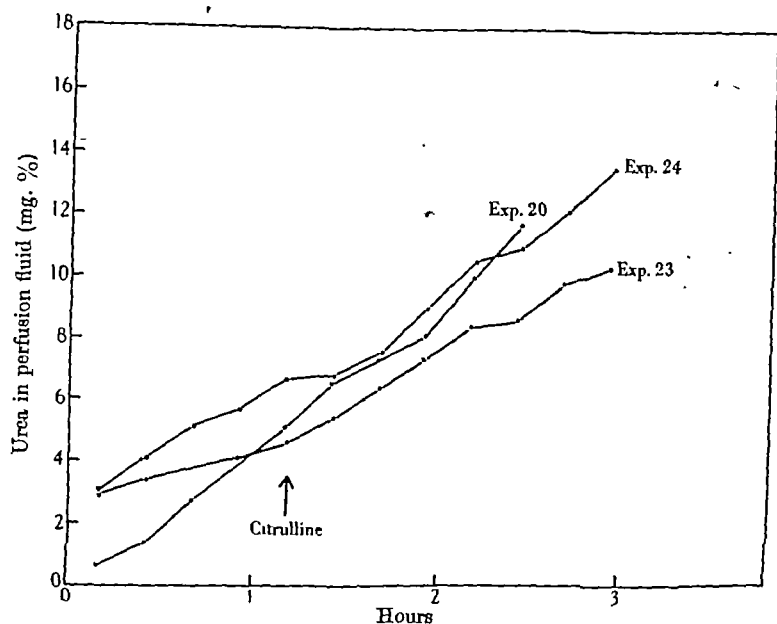


Fig. 11. The effect of citrulline in the presence of ammonia.

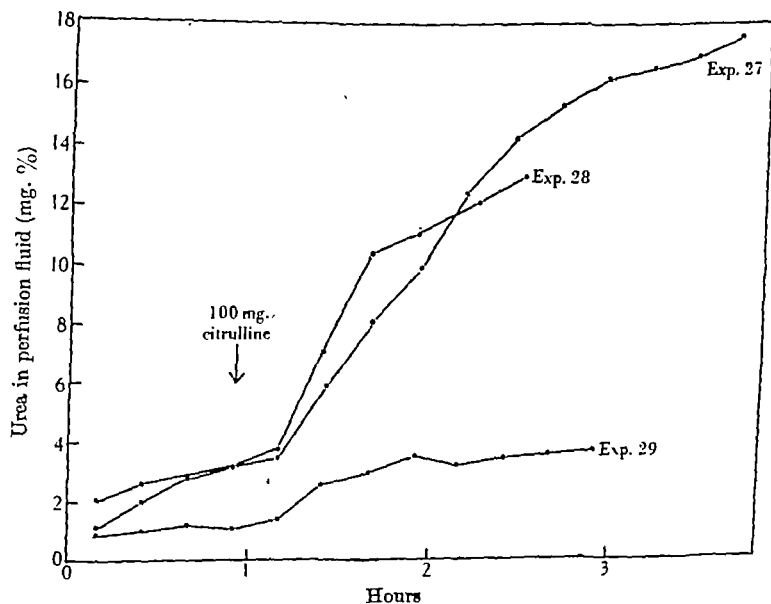


Fig. 12. The effect of a high concentration of citrulline. Exps. 27 and 28 in the presence of ammonia, Exp. 29 in the absence of ammonia.

interpretation. The increased urea formation with high concentrations of citrulline, found by Krebs & Henseleit and by Bach, is therefore confirmed, but it is shown that it is something quite different from the catalytic effects of ornithine and arginine.

Glutamic acid, glutamine, alanine

Two experiments with glutamic acid and two with glutamine showed that in concentrations of 10-20 mg. % neither of these substances had any effect on the rate of urea formation from ammonia. It was concluded that the amide nitrogen cycle of Bach does not operate at these concentrations, and that the effect of ornithine cannot be explained by its conversion to glutamine. Alanine, taken as a control amino acid, had no effect on the rate of urea formation from ammonia.

TABLE 2

Exp. no.	Dry wt. of liver g.	Mean total vol. c.c.	Perfusion flow c.c./min.	Ammonia conc. mg. %	Amino acid and conc. mg. %	Q urea in the presence of			
						Nil	Ammonia	Amino acid alone	Amino acid plus ammon
1	1.58	87	8.1-8.7	12.8	—	0	0.48	—	—
2	1.71	78	9.8-12.0	13.4	—	0	0.64	—	—
3	1.22	76	9.6	13.8	—	—	0.99	—	—
4	1.26	72	7.0-9.6	14.5	Ornithine 10.7	—	1.08	—	2.56
5	1.76	87	8.3	12.8	" 13.3	—	1.21	—	2.35
6	1.79	93	8.3-7.9	11.9	" 10.4	—	0.54	—	1.41
7	1.71	85	8.3	13.1	" 14.4	—	0.54	—	1.15
8	1.38	86	7.0-8.0	12.9	" 15.0	—	0.46	—	1.60
9	1.79	80	8.0	13.1	" 9.6	0.25	—	—	2.28
10	1.50	81	9.6	12.9	" 9.5	0	—	—	3.34
11	1.64	95	8.7-9.9	3.3	" 10.2	0	—	—	—
12	1.14	83	9.6-7.0	13.4	Arginine 12.0	—	1.16	—	4.00
13	1.43	87	10.2-12.2	12.8	" 18.8	0.59	—	2.52	2.57
14	1.32	82	9.0	—	" 20.0	0.26	—	5.47	—
15	1.15	86	8.0-9.0	11.1	" 19.1	0	—	7.10	2.11
16	1.81	86	9.2	—	" 19.1	0.15	—	0.94	—
17	1.47	81	8.0	—	" 24.7	0.13	—	0.52	—
18	1.66	82	8.3	12.8	" 14.6	—	—	0.35	1.32
19	1.66	93	8.0	10.2	" 12.9	0	—	0.61	1.58
20	1.59	80	6.9-8.7	—	" 15.0	0	—	0.59	—
21	1.08	82	6.9-9.2	13.5	Citrulline 24.4	—	1.31	—	1.31
22	1.56	83	8.8	13.4	" 15.0	—	0.73	—	0.73
23	1.07	85	8.0	12.3	" 11.6	—	—	—	1.15
24	1.72	83	8.3	13.4	" 14.4	—	0.30	—	0.61
25	1.48	85	8.3	13.1	" 14.1	—	0.81	—	0.74
26	1.21	79	7.0-8.4	14.1	" 16.4	—	1.24	—	0.78
27	1.41	85	8.3	13.1	" 14.1	—	—	—	1.24
28	1.98	95	8.3-11.8	11.7	" 105.0	—	0.35	—	—
29	1.27	87	6.6	12.8	" 115.0	—	0.73	—	—
30	1.42	90	8.3	—	" 111.0	0	—	—	—
31	2.28	93	8.9	11.9	Glutamine 12.5	—	0.37	—	0.37
32	1.67	94	8.3	11.8	" 12.3	—	0.32	—	0.32
33	1.51	81	8.0	13.7	Glutamic acid 12.4	—	0.40	—	0.40
34	2.44	97	7.3-7.8	11.4	" 15.5	—	0.30	—	0.30
35	1.37	80	8.0-9.6	13.9	Alanine 12.5	—	0.49	—	0.49

Additional notes

The fact that neither ornithine nor citrulline gave rise to any significant or sustained urea formation in the absence of ammonia indicates that these amino acids are deaminated very slowly, if at all, in the perfused liver.

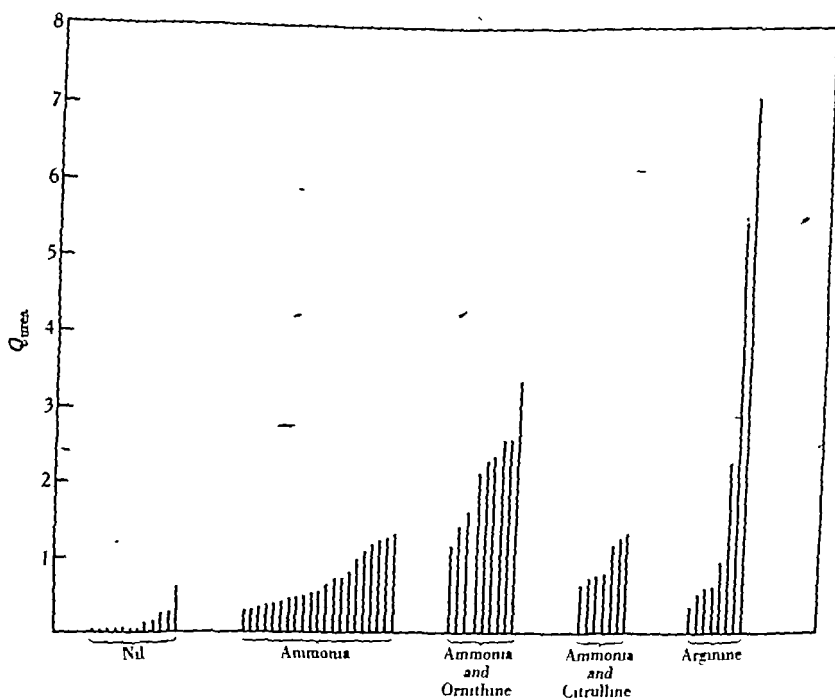


Fig. 13. Q_{urea} values in the presence of various added substances.

The addition of the various substances was not found to produce any alteration in the rate of perfusion flow. The figures given in Table 2 are for the maximum and minimum flows occurring during the experiment, where only one figure is given the flow was kept constant by adjusting the perfusion pressure.

In Fig. 13 the Q_{urea} values obtained under different conditions have been grouped so that a rapid comparison can be made. This figure illustrates in a different way several of the points which have already been demonstrated in individual experiments.

DISCUSSION

The most important fact which emerges, both from this work and that of others, is the catalytic effect of ornithine, and the main problem is to discover its significance. Krebs & Henseleit sought to explain it by putting forward their theory of an ornithine cycle. It is clear from the experiments reported here that this theory is, in its original form, untenable, for citrulline has no catalytic effect on urea formation and it is not converted into arginine. Also, the experiments with arginine showed that it is difficult to account for all the urea formation in terms of the ornithine cycle. The crucial point in the whole scheme is the conversion of ornithine to arginine. No evidence for this has so far been put forward, and such evidence as has been obtained here is against it.

Nevertheless, it is difficult to believe that the presence of arginase in the liver is entirely unconnected with the ornithine effect. The connexion might, however, be in the following sense. It may be that ornithine is normally necessary in the liver for a proper rate of urea formation from ammonia, and that the function of arginase is to furnish a continual supply of this catalyst. Ornithine does not occur in normal proteins, so any ornithine present in the body is presumably produced by arginase. In conditions of increased protein catabolism more arginine would reach the liver and correspondingly more ornithine would be formed in order to deal with the extra ammonia which would have to be converted to urea. There is nothing to support this speculation however, for although the normal presence of ornithine in the liver may be inferred from the presence of arginase and arginine, there is no evidence that ornithine is necessary for urea formation under normal conditions.

It must be concluded that both the normal mechanism of urea formation and the significance of the ornithine effect remain to be discovered.

SUMMARY

1. A method of perfusing the rat's liver with saline is described in which the whole apparatus is kept at 38° C., no metabolites can be lost from the circuit, the perfusion flow is constant for at least 3 hr. and is sufficient to ensure a good oxygen supply.
2. The method is designed for the purpose of metabolic experiments. The results are as accurate as those obtained by the tissue-slice method, and there is the further advantage that the rate of a metabolic process can be followed throughout the experiment.
3. The liver perfused by this method formed urea from ammonia at a rate which remained fairly constant for 4 hr.

4. The catalytic effect of ornithine on urea formation from ammonia was confirmed.

5. The rate of urea formation from arginine varied greatly from liver to liver, in some cases being much less than the rate of urea formation from ammonia in the presence of ornithine. This variation was not due to differences in arginase content. In livers in which the rate of arginine breakdown was slow, it was found that ammonia plus arginine gave a higher rate of urea formation than did arginine alone. These experiments show that the ornithine cycle cannot account for the whole of the urea formed from ammonia.

6. It was shown that citrulline has no catalytic effect on urea formation, and that it is not converted into arginine or ornithine.

7. In high concentrations, citrulline yielded a small quantity of urea and the yield was greater in the presence of ammonia.

8. Glutamic acid, glutamine and alanine had no effect on the rate of urea formation from ammonia, in the concentrations employed.

9. These results do not support either the ornithine cycle theory of Krebs or the amide nitrogen cycle theory of Bach. It is concluded that the normal mechanism of urea formation remains to be discovered.

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OLFACTORY REACTIONS IN THE BRAIN
OF THE HEDGEHOG

BY E. D. ADRIAN

From the Physiological Laboratory, Cambridge

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THOUGH many parts of the brain have been studied by recording their electrical activity there have been few attempts to apply this method to the olfactory regions. One reason may be that smell is not an important sense in man; another is that in mammals the olfactory parts of the brain are covered by the neopallium and are therefore difficult to expose. The difficulty is not insuperable, however, and varies from one species to another. The present work arose from the observation that in the hedgehog the olfactory bulb and the pyriform lobe are large and easily accessible and show a well-defined electrical activity related to olfactory stimulation. The same kind of activity has also been found in the cat. In several respects the olfactory discharge in the mammal resembles that found by Adrian & Ludwig [1938] in the olfactory tract of fish, though there are differences due to the periodic character of the stimulus and to a greater tendency to synchronization in the discharge.

METHOD

Material. Diagrams of the brain of the hedgehog (*Erinaceus europaeus*) are given in Fig. 1. There is a description of the olfactory regions by Retzius [1897] and a full account of the anatomy and cell structure of a closely related brain in a paper by Gray [1924] on the opossum (*Didelphys virginiana*). It will be seen that in the hedgehog the neo-cortex does not overlap the olfactory part of the cerebrum as in most mammals, but forms an incomplete cap below which the pyriform lobe bulges laterally. This part of the lobe can easily be reached by an electrode after the skull has been opened from above and the olfactory bulb is also very easily accessible.

Electrodes. In the earliest experiments one electrode was earthed and was attached to the tissues at the side of the head to form an indifferent lead. But with this arrangement it was found that the large potentials

developed in the pyriform lobe were bound to appear in the record whatever the position of the other electrode on the brain. For better localization, therefore, two electrodes were usually placed a few millimetres apart on the region to be examined. For leading from the surface the electrodes were tufts of cotton-wool held in a spiral of silver wire coated with silver chloride. When the interior of the olfactory bulb was to be examined, an enamelled silver wire was used instead of one of the cotton-wool electrodes. Both were mounted on adjustable arms on a vulcanite plate fixed to the skull with sealing wax [cf. Adrian & Moruzzi, 1939]. The potentials were recorded in the usual way with a Matthews oscillograph and loud speaker.

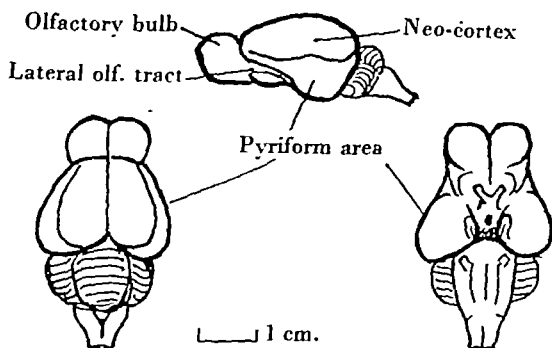


Fig. 1. Diagram of the brain of the hedgehog.

Anaesthetic. Most of the observations were made under nembutal anaesthesia of varying depth. Dial and chloralose were also used, the latter as an example of an anaesthetic with a different action from that of the barbiturates. Ether was unsatisfactory, as its use involved either the insertion of a tracheal cannula under another anaesthetic or else the exposure of the olfactory epithelium to ether vapour. Dial and nembutal certainly modify the activity of the olfactory pathways to some extent, but a comparison of different stages of anaesthesia shows that the modification is only one of degree.

Olfactory stimulation. No attempt was made to use accurately measurable olfactory stimuli, for the resulting activity cannot be measured accurately and there are too many uncertain factors, such as the amount of nasal secretion, the depth of anaesthesia and the rate of breathing. The method of stimulation originally employed was to place in front of the snout a small beaker containing an odorous solution or cotton-wool impregnated with an odorous substance. For stronger stimulation the

hedgehog was made to inspire through a wide glass tube which was fitted over the snout and lightly plugged with cotton-wool at the far end. A drop of tincture of asafoetida or oil of cloves could be placed on the cotton-wool at the appropriate moment. These simple methods were usually adequate, for with nembutal anaesthesia of medium depth reflex changes in breathing induced by the smell are rarely great enough to confuse the issue. But in the later experiments it was arranged that the air current through the nose should be independent of the depth of breathing. This was secured by fitting a tracheal cannula through which respiration took place and passing a second cannula upwards through the larynx and into the back of the nose (Fig. 2). The cannula was connected

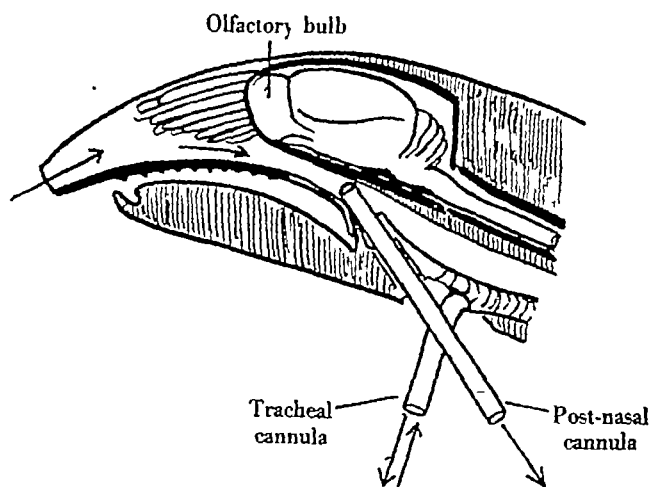


Fig. 2. Arrangement for drawing air through the nose independently of respiration.

with the suction side of a small respiration pump so that at each stroke a known volume of air was drawn through the nose. A minor trouble has been that in some animals the nasal secretions caused frequent blocking of the airway and consequent failure of stimulation unless the nose was cleared repeatedly.

RESULTS

Activity of the pyriform lobe

The response to air currents. The most prominent result is illustrated in the records in Figs. 3 and 4. These are made from animals under nembutal anaesthesia breathing quietly without any intentional olfactory stimulation. The periods of inspiration are marked above by a signal and it will be seen that at each inspiration there is a short group of potential

waves with a definite rhythm of 15-20 per sec. During the intervening period there are occasional waves at irregular intervals or there may be complete inactivity.

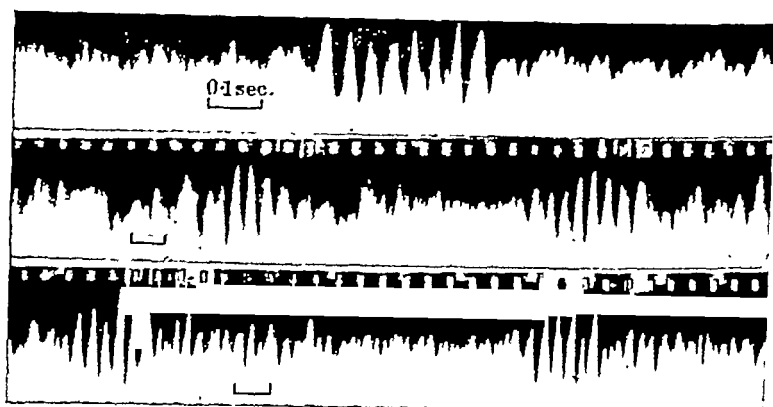


Fig. 3. Electrical activity of the pyriform lobe in animals breathing normal air. Records from three animals, all under nembutal anaesthesia. At each inspiration there is a series of regular potential waves at a frequency of 15-20 per sec. Horizontal line marks 0.1 sec. on each record. The large waves represent a change of 0.2 to 0.3 millivolts.

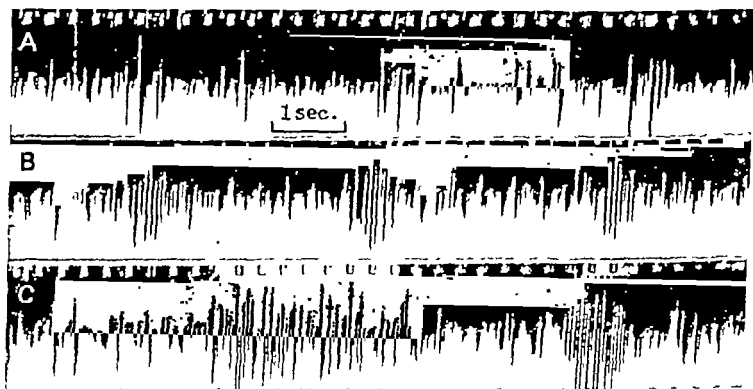


Fig. 4. Records from the left pyriform lobe in a hedgehog under nembutal. In A the left nostril was partially blocked with plasticene to reduce the flow of air through that side of the nose. In B the left nostril was free and the right was blocked. In C air was blown into the nostril from a glass syringe. Increasing the air current increases the number and frequency of the waves.

The regular wave groups are a true response of the olfactory system. They are not due to electrical spread from muscles or from parts of the brain directly concerned with respiration, for when the post-nasal

cannula is used the wave groups occur whenever air is sucked through the nose and bear no relation to the movements of breathing. If no air flows through the nose there are only occasional waves at longer intervals, and to produce the wave groups the air must flow through the nasal passages on the side under examination, for the groups are abolished by plugging the nostril on that side and increased by plugging the opposite side (Fig. 4 A, B).

But in spite of their olfactory origin the waves seem to depend more on the mechanical effect of the air current than on its smell. The velocity of the air through the nose is at any rate the principal factor in determining the rhythm. With very slow and shallow breathing there is some increase in the irregular waves during inspiration but no regular series; with deeper breathing the groups have a frequency which ranges from



Fig. 5. Hedgehog under nembutal. Air sucked through the nose by post-nasal cannula arrangement (Fig. 2). In A the air current is increased gradually and the wave frequency rises. In B the current is reduced and the frequency falls. Horizontal line marks 0.1 sec. in this and all subsequent records.

15 per sec. in deep to 25 in light anaesthesia, and when air is blown or sucked forcibly through the nose the frequency may rise as high as 35-45 per sec. These changes in the response are illustrated in Figs. 4 and 5.

The degree of regularity and the range of frequency of the waves varies from one animal to another. In some there is only a slight rise of frequency when the air current is greatly increased; in others (usually under lighter anaesthesia) the maximum frequency which can be produced by blowing is about twice that of the normal rhythm. The records in Fig. 5 are enough to show that the frequency of the regular wave discharge can certainly be varied by changing the flow of air through the nose. In Fig. 5 A for instance, a progressive increase in the air current makes the frequency rise from 16 to 30 per sec., and in Fig. 5 B a decreasing air current gives a progressive slowing. But it is often impossible to obtain a smooth variation over a wide range of frequencies. One reason

for this is that the air ways through the nose may be blocked by fluid and are freed suddenly when the air pressure rises. The stimulus is then a sudden blast of air and the response is always near the maximal frequency. Another reason may be the anaesthetic, for under dial only the lower frequencies can be obtained.

The regular wave rhythms are produced by air which has no smell distinct enough to be appreciated by the human nose. The air of a laboratory cannot be regarded as free from smell and may be highly odorous to

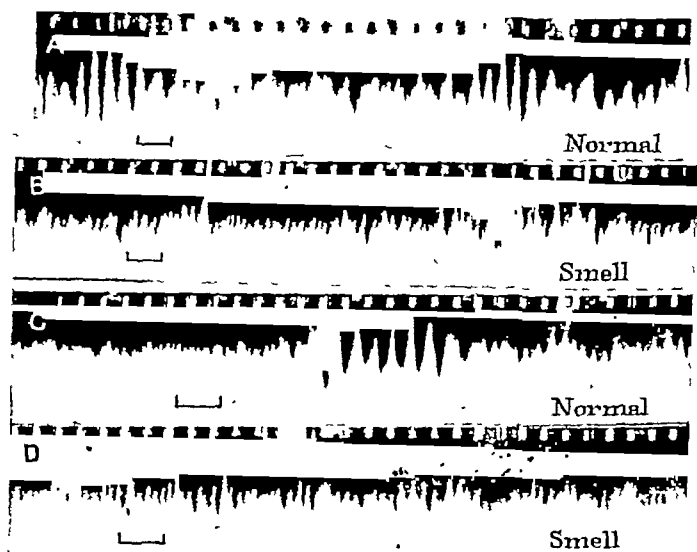


Fig. 6. The response to odours. Hedgehogs under nembutal. A and B from one animal. In A (normal air) regular waves appear in the pyriform lobes at each inspiration. In B the odour of asafoetida abolishes the waves. C and D from another animal; C breathing normal air, D breathing air with oil of cloves.

a macrosomatic animal; yet these regular responses seem to imply a uniform, synchronous activity which could scarcely be the basis for discriminative reactions to different smells. The way in which they are produced suggests instead that the waves represent a uniform mechanical stimulation by the air current and not a complex chemical stimulation by the odours in it. This view seems to be supported by the results of adding an odour strong enough to be detected by the human nose.

The response to odours. When a record is made from the surface of the pyriform lobe the result of adding a distinct odour to the air is to diminish or abolish the regular wave groups. In Fig. 6B for instance, a

but continuing throughout the whole respiratory cycle (Fig. 8). Here the slower synchronous wave response produced by the normal air current is converted into a rapid synchronous response by intense stimulation. It seems most likely, therefore, that the weaker stimulation causes an increase and not an inhibition of activity, and that the large waves are broken up because the different neurones are no longer all responding at the same frequency.

To decide the point we need to know whether there is in fact any increase in the discharge to the pyriform lobe when the large waves are abolished by an odour. It is not possible to record impulses in the non-medullated fibres of the olfactory nerve, but from certain parts of the olfactory bulb a wire electrode will pick up a characteristic activity and this gives most of the information we need.

Activity in the olfactory bulb

If an insulated silver wire is thrust into the olfactory bulb the crackling or rushing sound usually associated with a discharge of nerve impulses becomes audible when the wire has penetrated about 1.5 mm.

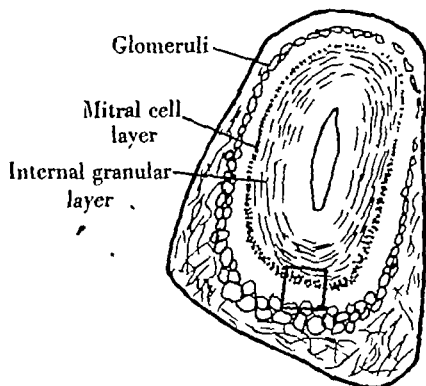


Fig. 9. Sketch of a section through the olfactory bulb to show the mitral cell layer and the region from which impulses can be recorded. The area stained by Hess's method is enclosed in a rectangle.

from the dorsal surface and again at a depth of about 5 mm. These two positions correspond with the layer of mitral cells and the axons which arise from them in the dorsal and ventral part of the bulb (Fig. 9). The localization was checked in one experiment by Hess's method [Hess, 1932]. An enamelled iron wire was used and when the active layer

was reached a current was passed through the wire to produce a deposit of iron in the tissues and this was afterwards stained with ferricyanide.

By manipulating the wire it is usually possible to obtain a record in which the potential changes are mostly brief axon spikes, though the amount of activity has always been too large to allow much inference as to the discharge in each unit. The activity consists of isolated bursts of impulses (or rather of potential spikes) between breaths with a more continuous discharge at each inspiration: and there is an immediate increase in the discharge if asafoetida or clove oil is brought near the nose.

The isolated bursts of impulses occur whenever there is a fairly long pause between each breath and the next. They are spaced rather irregularly at a frequency of 2-5 per sec. and they continue as long as the olfactory organ is intact. From some parts of the bulb indeed these irregular bursts are all that can be picked up, although from other parts there is a definite inspiratory effect as well. The bursts evidently correspond with the irregular waves recorded from the pyriform lobe and may be regarded as a resting discharge like that from other slowly adapting sense organs. Like other resting discharges they disappear for a few seconds after a period of increased activity, and for this reason they are absent when the breathing is moderately rapid and deep. It may be recalled that a considerable resting discharge was found by Adrian & Ludwig in the olfactory tract of the catfish. This also is abolished for a time following a period of increased activity.

When normal air (without intentional smell) is breathed the resting discharge quickens at each inspiration into a succession of outbursts at 15-20 per sec. (Fig. 10). These are evidently responsible for the regular waves in the pyriform lobe. When air is blown into the nostril or sucked through it by the post-nasal cannula the impulses are more definitely grouped into a series of volleys with a frequency of 30-40 per sec. Here also there are the regular waves in the pyriform lobe to correspond.

When clove oil or asafoetida is added to the air there is always an increase in the noise of the discharge at inspiration, and with a strong smell the increase may start before an inspiration has occurred. But in every case unless the smell is intense it has given a continuous stream of impulses rather than synchronised outbursts. Towards the end of the active period a grouping may appear again at a higher frequency if the smell is strong. Records illustrating the change from the grouped discharge of normal breathing are given in Fig. 11. They show that the disappearance of the regular wave response of the pyriform lobe is

not due to any lack of activity. With the specific olfactory stimulus the discharge from the olfactory bulb increases, but the increase is accompanied by a failure of the synchronization which was present when the air had no distinct smell.

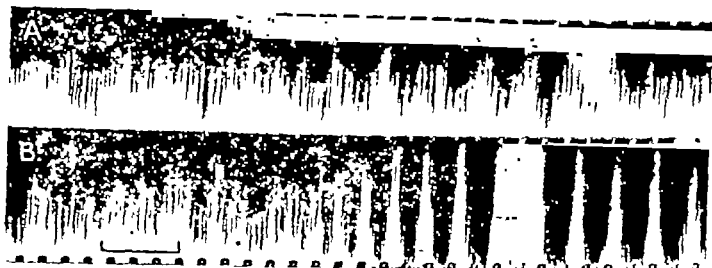


Fig. 10. Records from the mitral cell layer and fibres of the olfactory bulb. Hedgehog under nembutal. A, normal breathing. The bursts of the resting discharge quicken at inspiration to the 15 per sec. rhythm. B, blowing air into the nose. Synchronized volleys with an initial frequency of 30, falling to 20 per sec.

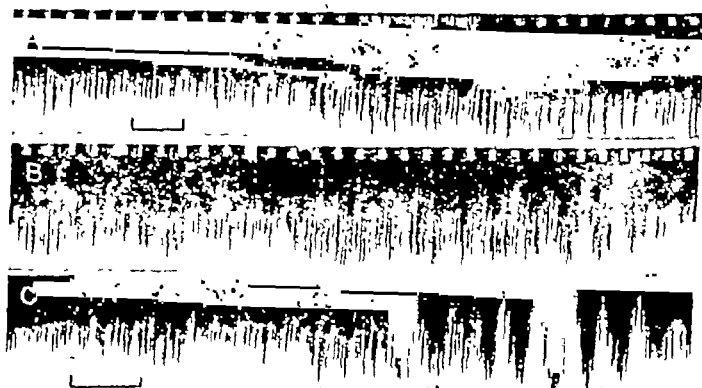


Fig. 11. Same animal as Fig. 10. Effect of adding a distinct odour. All three records show the effect of asafoetida. In A the discharge at inspiration is increased but there is no synchronization. In B and C synchronization begins towards the end of the discharge.

Substances causing an olfactory discharge

No attempt has been made to test a large number of odours. The substances regularly used were oil of cloves and tincture of asafoetida and these were always effective. Tincture of valerian, camphor and phenol were used occasionally and responses have been obtained with all three. Water in which an earthworm had been allowed to rot usually gave a large discharge when it was brought near the nose, but there was some-

times an inhibition of breathing to complicate the result. In all probability a smell with a weakly stimulating effect would not produce an obvious breakdown of the synchronized rhythm and would not cause any marked alteration in the olfactory bulb discharge unless the electrode happened to be favourably placed. Thus the method at present could scarcely be trusted to reveal threshold effects.

A point of some interest was the intense stimulation produced by tobacco smoke. If a small beaker is held in front of the nose and filled with cigarette smoke the discharge is much greater than that produced by asafoetida or clove oil and very soon becomes a regular series of volleys at a frequency of about 40 per sec. (cf. Fig. 12). This happens in an animal

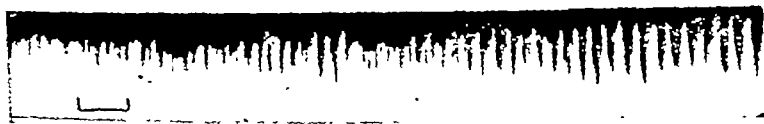


Fig. 12. Intense discharge produced by cigarette smoke. Hedgehog under chloralose with wire electrode in the olfactory bulb. Diffusion of smoke into the nose starts a series of volleys at 40 per sec.

breathing naturally although the synchronized discharge resembles that produced by blowing air forcibly through the nose: in some animals, moreover, the volleys may develop and persist although respiratory movements are completely inhibited. Thus an air current through the nose is not the only form of stimulus to give a synchronized discharge.

Olfactory responses in the cat's brain

Although only five successful experiments have been made on cats the results are enough to demonstrate that the olfactory response is of the same general character as in the hedgehog. To reach the lateral olfactory tract and the pyriform lobe, the eyeball was removed so as to expose the posterior wall of the orbit; a trephine opening over the frontal area was then enlarged downwards until the tract came into view. A cotton wool electrode resting on the tract will then pick up regular waves at each inspiration and the noise of impulses can often be detected by the loud speaker. The waves are presumably derived from the cortex of the pyriform lobe for they can be detected over a wide area whereas the impulse noise is confined to the tract. In three cats under nembutal the waves at inspiration had a frequency covering much the same range as in the hedgehog—from 12 per sec. in quiet breathing to 35 per sec. when air is blown into the nose. Under dial (two experiments) the frequency

has shown less variation. Asafoetida and clove oil have produced a considerable increase in the noise of the impulse discharge, but their effect on the regular waves is uncertain, for changes in the rate and depth of breathing have always occurred. Records from the cat's brain are given in Fig. 13. The resemblance to the hedgehog response is unmistakable, though more data will be needed for an accurate comparison.

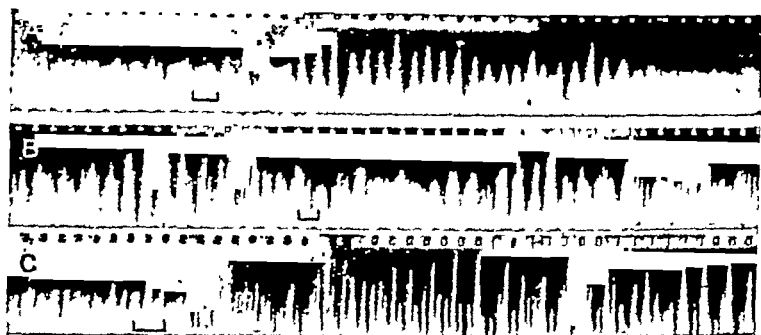


Fig. 13. Records from cats. Electrode on the surface of the lateral olfactory tract. A, cat under dial. Regular waves at 15 per sec. during normal inspiration. B, cat under nembutal. Large waves at 10 per sec. during inspiration. C, same animal as B. Increased activity due to cigarette smoke, maximum frequency 40 per sec.

DISCUSSION

It is interesting to find that even in deep anaesthesia there is a periodic activity in the olfactory part of the brain in phase with respiration. It was thought that it might perhaps be an important factor in maintaining the general level of excitation of the cerebrum, and that it might account for the periodic wave trains which appear in other parts of the anaesthetized brain. But in a cat under deep dial the waves which appear from time to time in, for example, the suprasylvian gyrus seem to be quite unaffected by procedures which would cause great modifications in the olfactory activity. Thus blocking the nose or blowing down it does not reduce or increase the periodic activity in the neo-cortex. In light anaesthesia these procedures certainly produce a widespread effect on the cortical waves, but clearly in deep anaesthesia the olfactory discharge has little to do with the periodic waves which occur in the non-olfactory areas.

Apart from this the main finding has been that an air current through the nose gives a response in the olfactory area made up of large regular waves, and that a smell converts this into small irregular waves. It is true that this statement needs some qualification; for instance, a rapid

air current will give large regular waves at a high frequency whether the air has a distinct smell or not, and an intense smell will give a regular high-frequency response on its own account. But there remains the point that over a considerable range an increase in the air current and an increase in the concentration of the odour have different effects, as though the air current tended to promote and the odour to prevent a synchronized discharge. To account for this it has been suggested that the olfactory receptors can be stimulated mechanically as well as chemically, and that the mechanical stimulation is more likely to be uniform.

The chief uncertainty arises from the fact that in these experiments the air passing through the nose could never be regarded as completely odourless. The regular waves found with normal breathing might therefore have been due to a weak chemical stimulation of the olfactory organ, the response becoming synchronized because with threshold stimuli the lowest frequencies would predominate. An explanation on these lines is scarcely possible, however, for it does not explain why an increase in the air current alone has never produced an irregular activity like that due to the addition of an odour. Instead it has given a regular response at a higher frequency. Another reason against the explanation is that the response to an air current is not modified in any way by the odours in it unless these are fairly strong. No doubt there is some olfactory stimulation by the odours from rubber tubes, from the operation wound or from the animal's own nose; none the less the waves in the pyriform lobe have been just as large when steps have been taken to reduce these odours as far as possible. Air which has been collected from the roof of the building and stored in clean glass vessels with no rubber connexions seems to have just the same effect when injected into the nose as air from the workroom. This absence of any obvious effect from weak odours is difficult to explain if the regular waves are set up by chemical stimulation; it is intelligible if we suppose that the effects of the weak odours are swamped by those of the air current acting as a mechanical stimulus.

It might be objected that we have no evidence from our own sensations that the olfactory organ can be stimulated by the movement of air over it. We do know, however, that the olfactory organ of the catfish responds to mechanical stimuli, for it responds to light pressure and to a current of water containing inert particles in suspension [Adrian & Ludwig, 1938]. Pressure on the olfactory organ has given a discharge in the hedgehog also, though it may have caused permanent injury. It is conceivable, therefore, that an air current might stimulate by bending

the hairs of the receptor cells, for it would be likely to cause some displacement in the layer of fluid and mucus covering the epithelium.

For the present then the view that there is normally a mechanical as well as a chemical stimulation of the olfactory organ seems to offer a reasonable explanation of the facts. In any case the facts leave no doubt that the excitation caused by a smell of moderate intensity is unevenly distributed and so tends to prevent a synchronous response; and from this it is a small step to the suggestion that the unevenness, i.e. the pattern of the excitation, will be different for different smells. It must be admitted that the present experiments give no direct proof that this is so. But their results are enough to support a comparison of the olfactory system with the visual. With both an intense stimulus gives a synchronized response at a high frequency, and with both a low-frequency rhythm, if it is present, is abolished by the kind of stimulus which leads to a discriminative reaction. A visual pattern abolishes the 'dark' rhythm of the eye of *Dytiscus*, or the resting rhythm of the striate area, because it forces the different units to respond at different frequencies, and a smell of moderate intensity abolishes the low-frequency response to an air current for the same reason. We recognize a sight not because particular receptors are stimulated but because a particular pattern of activity is aroused, and it is reasonable to conclude that we recognize a smell in the same way.

For a smell to produce a specific pattern of excitation in the olfactory epithelium we need only suppose that the different receptors are not all equally sensitive to different chemical stimuli. Such a differential sensitivity might depend on the intrinsic properties of the receptors, and it might also be due to extrinsic factors such as the amount of mucus in different regions, the rate of diffusion of the active molecules, etc. In this way an endless variety of smells might be distinguished because the process would be comparable not to the discrimination of colours but to that of visual patterns. It is to be hoped that further experiments will show whether this view can be confirmed.

CONCLUSIONS

1. In the hedgehog the olfactory bulb and the olfactory area of the brain are easily exposed and show a characteristic electrical activity in nembutal or chloralose anaesthesia.

2. Normal breathing produces a regular series of large potential waves in the pyriform area at each inspiration. These waves are due to the passage of air through the nose on that side. Their frequency varies from

15 per sec. during quiet breathing to 45 per sec. if the air is blown or sucked forcibly through the nose.

3. If a distinct odour (clove oil or asafoetida) is added to the air, the regular waves no longer appear at inspiration and their place is taken by small irregular waves. An intense smell may produce a continuous series of small waves at a high frequency (50 per sec.).

4. If a wire electrode leads from the mitral cell layer of the olfactory bulb, a discharge of impulses can be heard at each inspiration. The addition of a distinct odour increases the discharge. When air is blown through the nose the impulses become grouped into volleys at a high frequency.

5. These results are best explained on the assumption that the olfactory organ can be stimulated mechanically by the air current as well as chemically by the odours in it. The air current produces a uniform excitation which results in synchronous waves at a high or low frequency. The chemical stimulation is not uniform and so hinders the development of synchronous waves.

6. It is suggested that different chemical stimuli produce different distributions of excitation and that a familiar smell, like a familiar sight, is recognized by the specific pattern which it arouses in the brain.

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OBSERVATIONS ON THE PROPERTIES OF S-METHYL
ISO-THIOUREA SULPHATE, WITH PARTICULAR
REFERENCE TO THE CIRCULATORY EFFECTS

BY M. McGEORGE, M. SHERIF AND F. H. SMIRK

*From the Department of Medicine, University of New Zealand, Dunedin,
and the Department of Pharmacology, Egyptian University, Cairo*

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IN the course of an investigation of the properties of certain derivatives of thiourea the substance S-methyl iso-thiourea sulphate was observed by one of us (F. H. S.) to cause a rise of blood pressure when administered intravenously to dogs. The present paper reports some of the pharmacological effects of this substance in animals. A preliminary report has been published concerning its effects in man [Smirk, 1941].

METHODS

In experiments on the whole animal sodium barbitone was employed as an anaesthetic (average dose in dogs 0.1-0.3 g./kg., cats 0.2-0.6 g./kg., rabbits 0.6 g./kg.). Some of the cats and dogs were decerebrated at the level of the tentorium cerebelli, and a few were decerebrated and pithed, ether being used for anaesthesia during the operative procedures. The S-methyl iso-thiourea sulphate was injected in aqueous solution into a femoral vein. The resulting pressure changes in a carotid artery and in an external jugular vein were recorded by means of Messrs Palmer's arterial and venous pressure manometers respectively. The cannula in the external jugular vein was pushed nearly down to the auricle, so that free pulsations were visible in the manometer. When venous pressures were recorded coagulation of blood in the venous cannula was delayed by heparin. Electrocardiographic tracings were obtained, using the Cossor Robertson Oscillograph. The effects of S-methyl iso-thiourea sulphate when given after atropine or ergotoxine or after 1:3 methyl-piperidinobenzodioxan (F. 933) were studied in decerebrate cats and dogs. The effect of S-methyl iso-thiourea sulphate upon the blood pressure

was studied in experimental surgical shock, which was produced in anaesthetized dogs by complete abdominal evisceration, by crushing a limb, and by bleeding. The substance was also administered intravenously to unanaesthetized rabbits, and the blood pressure measured in the central artery of an ear by a modification of the method of Grant & Rothschild [1934].

The effect upon isolated organs was also studied. In the experiments upon the isolated heart of the rabbit and cat, oxygenated Ringer-Locke solution was perfused at a temperature of 37°C . through a cannula tied into the severed aorta. The effect upon the alimentary canal was studied chiefly by means of isolated rabbit intestine suspended in Ringer-Tyrode solution at 37°C . in the organ bath described by Burn & Dale [1922]. In a few cases Ringer-Locke solution was employed. A few experiments were also performed upon cat intestine, isolated bladder of the rabbit and cat, and the uterus of the rabbit, using the same apparatus. The effect upon the bronchioles was studied by the method described by Sollmann & Oettingen [1927], in which the bronchi are perfused with Ringer-Locke solution, the rate of drip from the cut surface of the lung providing a measure of the calibre of the bronchi.

RESULTS

The effect of intravenous injection of S-methyl iso-thiourea sulphate in cats, dogs and rabbits

Five experiments were carried out on decerebrate dogs, and in all cases a rise in blood pressure following intravenous injection was recorded. In one of these experiments the following result was obtained:

The blood pressure, which was initially 120 mm. of mercury, fell spontaneously to 58 mm. A dose of 0.1 g. of S-methyl iso-thiourea sulphate was then administered to the dog, which weighed 7 kg., and the pressure rose to 192 mm., falling in the course of 12 min. to 120 mm., and 20 min. later to 98 mm., at which level it remained for at least 72 min. The pressure in the external jugular vein was approximately zero before the administration, fell by 2 cm. of water during the acute rise of blood pressure, and returned to about the original level after 20 min. Other results obtained in decerebrate dogs, and also in dogs anaesthetized with sodium barbitone, were essentially similar to this one, but with somewhat smaller blood-pressure increases.

Six observations were made on decerebrate pithed cats after a sufficient lapse of time to permit of recovery from the ether anaesthesia.

Intravenous injections of S-methyl iso-thiourea sulphate caused blood-pressure increases in all these animals (Fig. 1). Evidently the blood-pressure rise does not depend upon the integrity of the central nervous system. In one experiment 0.1 g. of the substance raised the blood pressure from 30 to 155 mm. This was followed by a gradual fall to a level of 60 mm. after 45 min. In another experiment three successive

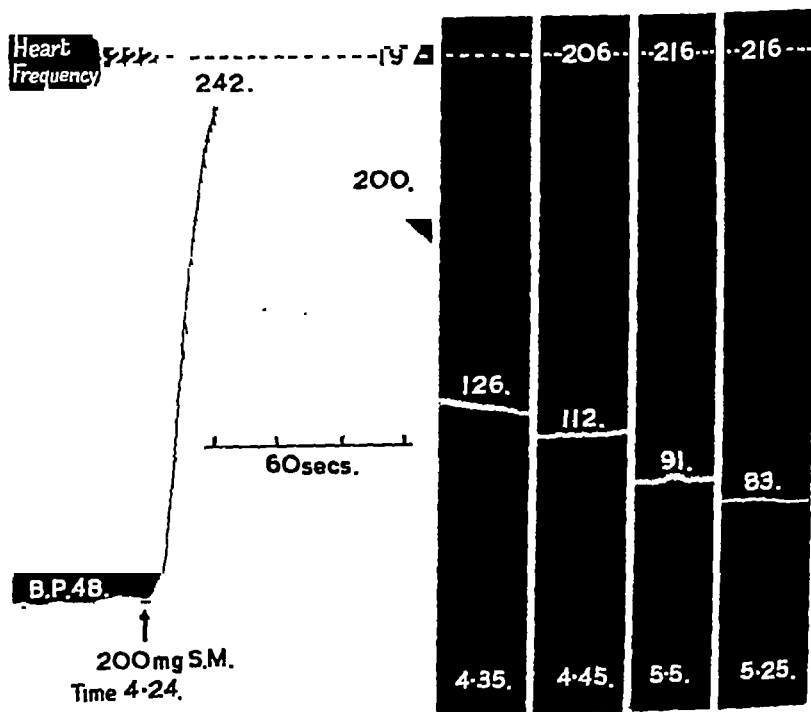


Fig. 1. Effect on blood pressure and heart frequency of decerebrate pithed cat following 53 mg./kg. S.M. (Figures written along the tracing represent the blood pressure.)

injections of 0.2 g. caused blood-pressure increases of 94, 60 and 68 mm. respectively. Over a period of 3 hr. another cat received in all 2.1 g. of the substance, with repeated blood-pressure rises, but with no obvious ill effects. It was observed that when repeated injections were given, each containing the same quantity of S-methyl iso-thiourea sulphate, the blood-pressure rises from the later injections were smaller than those resulting from the earlier injections.

Results similar in type, but with less pronounced blood-pressure rises, were obtained in two experiments on rabbits under sodium bar-

bitone anaesthesia. In one rabbit 0.01 g. raised the blood pressure by 26 mm. to a level of 88 mm., after which a dose of 0.02 g. produced a further rise to 105 mm., at which the pressure remained for 5 min. The blood pressure was raised above the initial level for a period of 30 min. Doses of between 0.025 and 0.05 g. given intravenously to unanaesthetized rabbits caused prompt blood-pressure increases in four out of five rabbits. The blood-pressure rise was between 25 and 30 mm. of mercury, and some increase in the blood pressure usually persisted for an hour or more. These rabbits were all alive at the end of 3 months. In three other instances 0.1 g./kg. given intravenously caused death with convulsions.

The rise in blood pressure ordinarily elicited by the intravenous injection of S-methyl iso-thiourea sulphate was not prevented in dogs by the previous administration of atropine (about 1 mg./kg. dog). Ergotoxine was given to four decerebrate dogs and six decerebrate cats until they responded to a dose of adrenaline by a fall in the blood pressure. Each cat then received several separate injections of S-methyl iso-thiourea sulphate, varying in amounts from 0.0025 to 0.05 g., the dogs receiving injections of about 0.1 g. In this series of twenty-six injections of the compound, administered to ten animals which had been previously treated with ergotoxine, the blood-pressure increase (Fig. 2) was obtained twenty-four times. A blood-pressure fall, however, was obtained following two injections, and was due apparently to excessive slowing of the heart. In experiments on three cats the substance 1:3-methylpiperidinobenzodioxan (F. 933) was administered in doses sufficient to inhibit the blood-pressure raising action of adrenaline. The pressor effect of S-methyl iso-thiourea sulphate remained unimpaired (Fig. 3), a rise in the blood pressure occurring each time with six injections. These experiments show that the blood-pressure raising action of S-methyl iso-thiourea sulphate is not prevented by paralysis of the motor effects of the sympathetic, either by ergotoxine or by F. 933.

In four experiments S-methyl iso-thiourea sulphate was administered to anaesthetized dogs in which profound shock had been induced by procedures such as crushing of limbs, complete evisceration, and bleeding. Prior to the administration the blood pressure had fallen to between 20 and 30 mm. of mercury, and death would undoubtedly have ensured in a short time had not the circulation been maintained by repeated injections of S-methyl iso-thiourea sulphate. In one instance the blood pressure rose from 30 to 140 mm., and in all these experiments the animal was maintained alive for several hours, and further operative procedures were undertaken without causing circulatory collapse. Individual doses

ranged from 0.1 to 0.2 g., but as much as 2 g. was given in the course of 2 hr. without causing any obvious impairment of the circulation. The

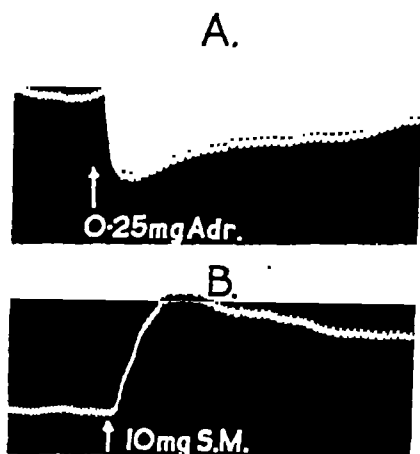


Fig. 2. Effect of ergotoxine on the pressor effect of S.M. in a decerebrate cat. A. Blood-pressure fall from adrenaline given after ergotoxine. B. Pressor effect of 2.8 mg./kg. S.M. given after the adrenaline reversal by ergotoxine.

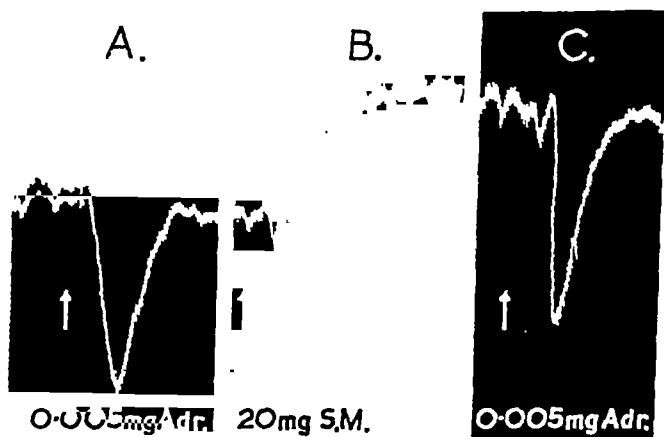


Fig. 3. Effect of F. 933 on the pressor effect of S.M. in a cat anaesthetized with sodium barbitone. A. Blood-pressure fall after adrenaline following intravenous injection of 0.8 mg./kg. F. 933. B. Pressor effect of 8 mg./kg. S.M. following adrenaline reversal by F. 933. C. Persistence of adrenaline reversal after administration of S.M.

experiments on pithed cats already described also provide evidence of the effect of the compound in states of circulatory collapse.

The effect of S-methyl iso-thiourea sulphate on isolated organs

The effect of S-methyl iso-thiourea sulphate on the circulatory system was further studied in a series of nine experiments on the isolated heart of the rabbit. It was found that concentrations of 1/500,000 in Ringer-Locke solution usually caused a slight decrease in the amplitude of contraction. Higher concentrations led to an appreciable decrease in the amplitude of contraction, associated with slowing of the heart, and sometimes complete heart block. The previous addition to the perfusion fluid of atropine in concentration of 1/100,000 did not eliminate the heart slowing action of S-methyl iso-thiourea sulphate (Fig. 4). It follows

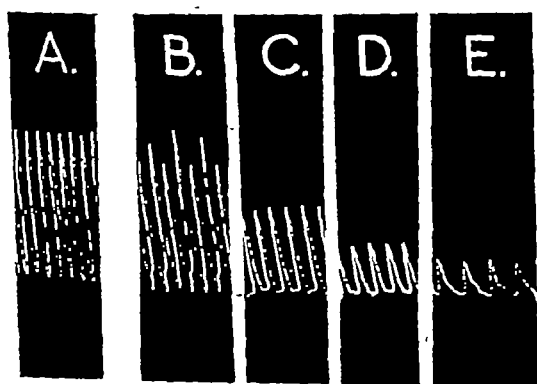


Fig. 4. Effect of a concentration of 1/200,000 S.M. upon the isolated heart of a rabbit, perfused with Ringer-Locke solution containing 1/100,000 atropine. A. Before addition of S.M. B, C, D, E. Sections of tracing at intervals of 1.6, 4.5, 7.7, and 21 minutes after addition of S.M., showing slowing and decrease in amplitude of contraction.

therefore that at least an important part of this action does not originate in the vagal system. Two experiments of similar type were also made on the isolated heart of the cat. In one of these a concentration of 1/100,000 of the substance in Ringer-Locke solution decreased the amplitude of contraction of the heart by approximately one-half, and the rate from 180 to 98. The amplitude of contraction recovered fully on return to Ringer-Locke solution. A concentration of 1/5000 then produced a sharp decrease in amplitude, succeeded by heart block, and on return to Ringer-Locke solution normal rhythm did not reappear. An essentially similar result was obtained in experiments on the heart of the second cat.

Further observations on the slowing of the heart by poisonous doses of S-methyl iso-thiourea sulphate were made in unanaesthetized dogs by

electrocardiographic study. Some striking changes in the electro-cardiogram were observed, there being a notable increase in the height of the *T* wave, and also of the *R* wave. In some experiments the contractions of the auricles ceased, and the contractions of the ventricles appeared to originate from an ectopic focus. Sometimes complete heart block was present, and occasionally a marked increase in the *PR* interval without complete block. Inversion of the *T* wave was observed as the effect was subsiding.

It has been shown that the action of S-methyl iso-thiourea sulphate in raising the blood pressure is independent of the central nervous system, and there is some evidence suggesting that it is the result of

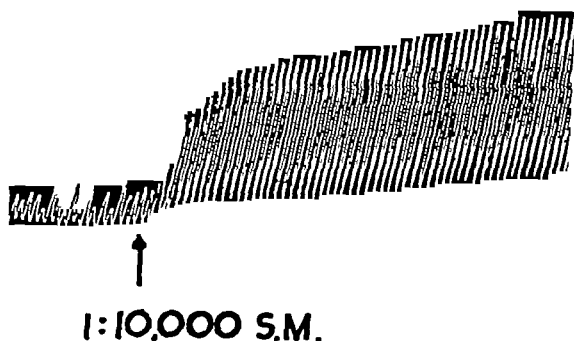


Fig. 5. Effect of S.M. (1/10,000) on rabbit intestine suspended in Ringer-Tyrode solution containing 1/100,000 atropine.

a direct action upon the smooth muscle of the blood vessels. In view of the vaso-constriction set up by the compound it is of interest to study its effects upon other smooth muscles, such as that of the intestine, urinary bladder, uterus and bronchioles.

In a concentration of 1/600,000 this compound produces a definite increase in the tone and strength of contraction of rabbit's intestine suspended in Ringer-Tyrode solution. Atropine in a concentration of 1/100,000 does not prevent the action of a concentration of 1/10,000 of S-methyl iso-thiourea sulphate (Fig. 5), while 1/10,000 atropine causes a considerable diminution in contraction from 1/10,000 S-methyl iso-thiourea sulphate.

The bladder is much less sensitive than the intestine to S-methyl iso-thiourea sulphate, but responds to a concentration of 1/20,000 by a

slow, prolonged increase in muscle tone (Fig. 6). This also persists in the presence of atropine. In three experiments, also, strong contractions of the pregnant uterus were obtained (Fig. 7).

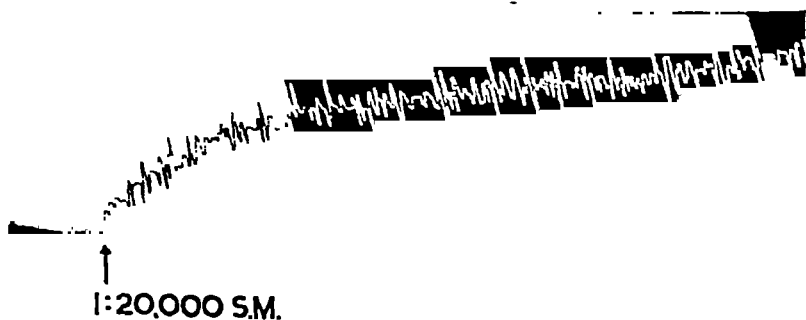


Fig. 6. Effect of S.M. (1/20,000) on isolated rabbit bladder.

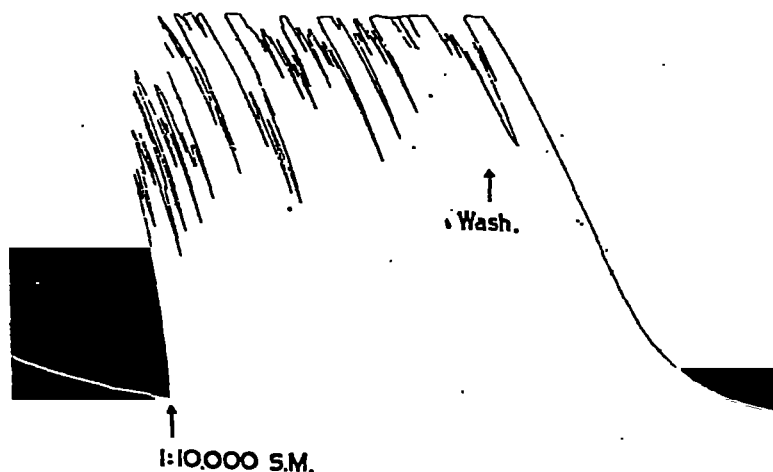


Fig. 7. Effect of S.M. (1/10,000) on isolated pregnant uterus of the rabbit.

The effect upon the muscle of the bronchioles has been studied, but no striking effect was observed, even in concentrations which must have been in the region of 1/5000 or stronger.

DISCUSSION

S-methyl iso-thiourea sulphate when injected intravenously into animals produces a considerable rise of blood pressure, accompanied usually by a slight degree of heart slowing. The present investigation is concerned mainly with the mechanism of these effects.

Intravenous injection of S-methyl iso-thiourea sulphate into the pithed cat may raise the blood pressure from 30 mm. to as high as 260 mm., an effect which cannot be due to an action on the central nervous system, since the whole of this has been removed. It is highly probable also that the site of its action is not in the peripheral part of the nervous system either. In animals in which the sympathetic motor nerve endings have been blocked by either ergotoxine or F. 933, adrenaline has no blood-pressure raising action, whereas S-methyl iso-thiourea sulphate still causes striking blood-pressure increases. This evidence, while not conclusive, suggests that the effect of the substance is directly-upon the plain muscle of the blood vessels.

The heart slowing, when it occurs, is not the result of any central stimulation of the vagal system, since it is present in the pithed cat and when the vagi are divided. The substance also causes slowing of the isolated heart, whether perfused by normal or by atropinized Ringer-Locke solution. It is highly probable, therefore, that an important part of the heart slowing action is not of peripheral vagal origin, and most likely results from some direct effect upon the sino-auricular node, conducting tissue, or heart muscle. Most of the substances employed in therapeutics which cause slowing of the pulse do so by stimulating some part of the vagal system, and in several instances the decrease in heart rate is accompanied by a fall in the blood pressure. S-methyl iso-thiourea sulphate appears to be exceptional in that both the blood-pressure increase and the bradycardia which it causes seem to be the result of an action on muscle.

The effects upon the heart in the whole animal and upon the isolated heart differ, however, in one important particular. A concentration of 1/500,000 may cause slight depression of the isolated heart, while a concentration of 1/50,000 is likely to cause marked slowing, followed by complete heart block, and later arrest of the heart. But in striking contrast to this is the fact that when the concentration in the blood of a whole animal, cat or rabbit, must be at least ten times, and may even be fifty times, greater than this, the degree of slowing of the heart is relatively slight, and heart block does not occur. Hence it is possible, but

not proved, that in the whole animal the substance undergoes a change in composition which does not take place when it is perfused through the isolated heart. Further observations on this point are required.

S-methyl iso-thiourea sulphate in a concentration of 1/600,000 stimulates contraction of small strips of isolated rabbit intestine suspended in Ringer-Tyrode solution. The same stimulation of the intestine is obtained in whole animals, though the dose required to cause defaecation is greater than that required to produce strong circulatory effects. Were the substance a sympathetic stimulant it would tend to relax intestine. When the isolated intestine is suspended in Ringer-Tyrode solution containing 1/10,000 atropine the substance causes slight contraction of the intestine. Where the atropine concentration is 1/100,000, S-methyl iso-thiourea sulphate (1/10,000) causes strong contraction. Similarly the isolated muscle of the bladder and uterus is also contracted.

SUMMARY

1. The pharmacological action of S-methyl iso-thiourea sulphate has been investigated in animals.
2. When injected intravenously in anaesthetized, decerebrate, and pithed animals, it produces a considerable and prolonged rise in blood pressure, accompanied usually by some slowing of the heart.
3. The blood-pressure rise is still obtained when the motor effects of the sympathetic have been eliminated by ergotoxine or by 1:3 methyl-piperidinobenzodioxan (F. 933).
4. The substance depresses and slows the atropinized perfused heart of the rabbit and cat.
5. The substance causes contraction of isolated strips of smooth muscle from the intestine, bladder and uterus. The effect on the intestine is diminished, but is not abolished, by atropinization.
6. The central nervous system is stimulated and respiration increased, but most of the effects recorded did not depend upon the integrity of the nervous system.
7. The blood pressure is raised in anaesthetized animals suffering from severe surgical shock produced experimentally. It has been shown that the substance stimulates most smooth muscle by a peripheral action, and there is some evidence suggesting that the effect is directly upon muscle.

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Observations on adreno-corticotrophic action. By Y. M. L. GOLLA and M. REISS (introduced by J. H. GADDUM). *From the Burden Neurological Institute, Stoke Lane, Stapleton, Bristol*

In assaying anterior pituitary lobe preparations for adrenocorticotrophic activity, use has been made, on the one hand, of the power to promote increase of weight of suprarenals in hypophysectomized rats [Collip, Anderson & Thomson, 1933] or two-day-old chicks [Bates, Riddle & Miller, 1940], and, on the other, of the effect in restoring the histological appearance of the adrenal cortex, with disappearance of the sudanophobe zone in the zona fasciculata, which invariably is seen after hypophysectomy [Reiss, Balint, Oestreicher & Aronson, 1936].

It has hitherto been assumed that those two actions were due to a single 'corticotrophic' factor. It has now, however, been found that there is present in the serum of pregnant mares a substance which, on injection, is capable of increasing the weight of the suprarenals, or checking further atrophy in hypophysectomized animals without affecting the sudanophobe zone. Similar effects may occasionally be obtained with the serum of other (non-pregnant) animals (rabbits, rats). This substance is not identical with the gonadotrophic factor, since it survives the heat treatment which entirely destroys the latter.

The question therefore arises whether there are not at least two corticotrophic factors, (a) regulating the size of the cortex, and (b) regulating lipid formation in the cortex. Such a conception would explain discrepancies experienced in the assay of anterior pituitary lobe preparations by the sudanophobe zone test, where the histological picture was judged to be restored, but the effect on the weight of the glands was found to be irregular.

Additional evidence of the dual character of corticotrophic action is supplied by experimenting on the effects of injection of oestrone on compensatory adrenal hypertrophy following unilateral adrenalectomy.

With hypophysectomized animals, no hypertrophy occurs (absence of corticotrophic factor production), whereas in normal animals the resultant hypertrophy averages about 60 % weight increase, following oestrone injection an increase up to 250 % or more may be observed. In the former case, the lipoid appears normal, in the latter it is invariably much reduced and may even disappear.

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Speeding-up of cell divisions of macrophages *in vitro*. By F. JACOBY. *From the Department of Physiology, The Medical School, Birmingham*

Serial photographic studies of chicken macrophages *in vitro* have shown that when a population of these cells is, from a quiescent state, stimulated to activity by serum feeding, a wave of cell divisions sets in usually after a latent period of 20–24 hr. [Jacoby, 1937, 1938]. The time taken for division by the individual cell, estimated on a large number of mitoses, averages 34·2 min.

Subsequently I found that when two successive serum feedings are given at an interval of 20 hr. the latent period following the second feeding was—as should be expected—considerably shortened (Table 1). Further analysis of the photographs (paper films) revealed that the cell divisions also were significantly speeded up, the average time being 26·5 min., and that in a high percentage of these mitoses the cells did not round off completely, as is usually the case, but remained elliptical or more frequently even spindle shaped. In frequency curves of the duration of the mitoses the peak for the experimental cultures (comprising 50 % of the divisions) lies at 24 min., for the controls at 36 min.

TABLE 1

	Length of latent period hr.	Av. duration of mitosis min.	No. of cells incompletely rounding off %	No. of mitoses studied
Exp. TM 53	14	26	42	148
Exp. TM 55	3	27	44	434
Controls	20–24	34·2	0–16	1238

The two successive feedings applied bring the cultural conditions somewhat closer to the conditions which are present *in vivo*. The findings,

therefore, make it likely that the cells may behave similarly in the organism wherever their activity is stimulated.

For some time a higher rate of multiplication went parallel with the shortening of the duration of mitosis, but counting longer time stretches the average rate was 2-3 % per hour which is in agreement with studies on liver regeneration [Brues & Marble, 1937], on the growing heart in early embryonic life [Olivo & Slavich, 1929] and with tissue culture studies on fibroblasts [Olivo, 1931; Willmer, 1933].

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Alkali denaturation of oxyhaemoglobin, haemoglobin, carbon-monoxymhaemoglobin, methaemoglobin and cyanmethaemoglobin. By H. S. BAAR and E. M. HICKMANS (introduced by H. P. GILDING). *From The Children's Hospital, Birmingham*

The spectrophotometric method of Haurowitz has been adapted for the use of Evelyn's photoelectric step photometer in these investigations. The denaturation product is alkaline globin haematin. Kathaemoglobin bands appear only after neutralization with CO_2 or CH_3COOH . If globin haemochromogen is shaken with air at pH 12-13 alkaline globin haematin results and kathaemoglobin bands are absent. Globin haematin from sheep's haemoglobin separated from stromata shows the same absorption curve as that from R.B.C. and the same as the serum haematin compound. Saponin alters the haemoglobin molecule. Both haemoglobin and globin haematin follow the Beer-Lambert law. The observation of Brinckman that the denaturation curve of adult's oxyhaemoglobin shows a decrease in velocity has been confirmed. But while the denaturation curves of HbO_2 from young babies determined on a series of subsequent days show a remarkable constancy, in the case of adult's HbO_2 only the first part of the curve is constant, whereas in the second part considerable variations occur even in the same blood sample. Occasionally a perfectly straight line is seen. Babies between 8 months and 2 years show the same type of curve as adults. The retardation is due to an inhibitory action of

the reaction products and to absorption of CO_2 . Occasionally a denaturation curve with retardation yields a straight line when plotted on bilogarithmic scale. The amount of alkali resistant HbO_2 in young babies decreases slowly even in cases with high destruction and regeneration rates. Resistant haemoglobin is therefore formed in extrauterine life. Denaturation curves of HbO_2 , Hb, Met-Hb, and CNMet-Hb show small differences, the denaturation velocity of HbCO is much lower than that of HbO_2 . Denaturation of CNMet-Hb yields alkaline globin haematin which only after further addition of NaCN is transformed into CN-haematin.

The quantitative determination of alkali resistant and labile HbO_2 has been used to follow the fate of transfused R.B.C. in icterus gravis and erythronoclastic anaemia of the newborn. Two types have been found. In one type the rise in the amount of labile HbO_2 following a transfusion remained unchanged for a long time despite a high destruction and regeneration rate. In the second type the amount of labile HbO_2 decreased rapidly within 2 days to the pretransfusion level. Intermediate cases have been observed. The indiscriminate destruction of donor's and recipient's cells in the second type is evidence of a haemolytic factor (Parsons and his school). The selective destruction in cases with less violent haemolysis indicates that the R.B.C. are already damaged when entering the circulation. The noxious agent affects the whole erythron and the anaemia is really erythronoclastic (Parsons) in origin.

The bilirubinoid pigments of human faeces. By H. S. BAAR and E. M. HICKMANS (introduced by H. P. GILDING). *From the Children's Hospital, Birmingham*

The study of absorption curves of more than 2000 acid alcoholic stool extracts, combined with spectroscopical and fluoroscopical examination, revealed the presence of at least one pigment besides stercobilin, copromesobiliviolin and copronigrin. Incubation of stool emulsions showed a mutual transformation of stercobilin and a pigment or pigments with increasing light extinction between 490 and 420 $\text{m}\mu$. Crystalline stercobilin kept at room temperature shows an increase of the A value and simultaneously a decrease of the $\text{E}_{490}/\text{E}_{440}$ quotient. Spectrophotometric examination of various fractions and residues during the preparation of crystalline stercobilin confirmed the results of the analysis of stool extracts. By fractional extraction and chromatographic separation two

pigments have been isolated from human faeces: a red crystalline substance soluble in petrol ether (stercorubrin) and a brown amorphous substance insoluble in petrol ether (stercofulvin). These pigments have a similar absorption curve between 490 and 420 $m\mu$ but differ in the spectral region 520–490 $m\mu$. Both show a reddish yellow fluorescence. Absorption curves of stool extracts indicate that either one of these pigments is always prevalent or that both run parallel. This justifies a photometric estimation on the basis of a two pigments mixture. 'Stercobilin' (Garrod and Hopkins' method, modified by Elman and McMaster) is an impure mixture of stercobilin and stercofulvin (and/or stercorubrin). Stercifulvin and stercorubrin have not yet been obtained in pure form. The quotient E_{490}/E_{440} could therefore be determined only (0.45) and not the A value. For pure crystalline stercobilin (Watson) in Elman and McMaster's diluting fluid an A value of 1.86×10^{-5} at the maximum has been found. With the use of Evelyn's photometer an A value of 3.6×10^{-5} was found for filter 490 and 15.1×10^{-5} for filter 440. A values have been determined for crystalline mixtures of stercobilin and stercofulvin (and/or stercorubrin). With these figures and the known E_{490}/E_{440} quotient the A value has been calculated for stercofulvin (and/or stercorubrin) by means of the Vierordt formula. Using Evelyn's photometer this A value for filter 490 is 1.025×10^{-3} and for filter 440, 4.62×10^{-4} . Using the Vierordt formula the A values have been calculated for a series of pigment mixtures and plotted against the E_{490}/E_{440} quotients. The resulting regular curve allows an easy calculation of 'total bile pigments' if the light extinctions of an acid alcoholic stool extract diluted with Elman and McMaster's diluting fluid are determined with the filters 490 and 440. Copromesobiliviolin is not included in this estimation. Appreciable amounts of this pigment are not often found.

On the use of perspex to form a surface on which the coagulation of blood is delayed. By F. SCHÜTZ (introduced by J. H. GADDUM).
Pharmacological Laboratories, Pharmaceutical Society of Great Britain

Perspex is a glass-clear artificial resin, from which vessels can be made, in which the coagulation of blood is delayed. Blood was allowed to run through a cannula from the carotid of a rabbit on to a watch glass, and on to a similarly shaped vessel made of perspex. On glass it clotted in 10 min., but on perspex it only clotted after 35 min. Clotting was also delayed when blood was collected in test-tubes, made of perspex by

pouring a freshly prepared 5-10 % solution of perspex in water-free pure acetone into glass test-tubes and then pouring it out again, as when collodion bags are made for dialysis. The perspex retracts slowly from the glass tube, which should be rotated for about 12 hr. when a thick layer has been applied. When completely dry, the perspex tube can be easily removed. In three experiments (two spinal cats and one rabbit anaesthetized with pernocton) blood clotted after the same time in these perspex tubes as in glass tubes lined with paraffin. The perspex vessels have the advantage of being transparent; it is known that perspex absorbs less visible and invisible light than glass.

Attempts have been made to line arterial cannulae with perspex, but it is difficult to avoid retraction of the perspex from the glass at points where the diameter of the tube is changing rapidly. Vessels of almost any shape can, however, be made of perspex by a mechanic, as perspex is easily cut and shaped. Thick centrifuge tubes were made by repeatedly lining glass tubes with the solution. These have the advantage that they are less easily broken than glass tubes.

The clotting of recalcified oxalated blood is not delayed on perspex.

I should like to express my gratitude to Prof. J. H. Gaddum and to Dr H. H. Hamburg of Combined Optical Industries, Slough, for valuable advice and interest and to Imperia Chemical Industries (Plastics) for kindly presenting me a quantity of perspex.

PROCEEDINGS

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Initiation of lactation in nulliparous heifers by diethylstilboestrol. By S. J. FOLLEY, H. M. SCOTT WATSON (Mrs C. C. THIEL) and A. C. BOTTOMLEY. (*National Institute for Research in Dairying, University of Reading*)

Experiments on the induction of lactation in virgin goats by diethylstilboestrol have been reported in these Proceedings [Folley, Scott Watson & Bottomley, 1940]. Similar experiments were begun in 1939 on two nulliparous Dairy Shorthorn heifers. Mammary secretion was initiated in these at respective ages of 18½ and 20 months by inunction of the udder with ointment containing diethylstilboestrol. Daily milking for prolonged periods previous to treatment produced a small amount of a clear fluid in one case and nothing in the other. In the first case application of 5.0 g. of 1% diethylstilboestrol dipropionate ointment three times weekly was quickly followed by secretion of an opaque fluid, the yield of which rose to 80 ml. daily and then dropped sharply. The yield remained at about 20 ml. daily during a period of inunction with inactive ointment but rose again when half the original dose of active ointment was used. When inunction was stopped the yield rose further to a peak of about 170 ml. daily and then slowly declined as in a normal lactation. Essentially similar results were obtained with the other heifer.

During the period of oestrogen treatment in both experiments, the yield of secretion showed regular fluctuations correlated with the oestrous cycle, minima occurring at or near oestrus and maxima about 7 days later. Evidently at oestrus there was sufficient oestrogen in the body, endogenous and administered, to inhibit lactation. The composition of the secretion varied somewhat at different periods of the experiment but never resembled that of normal milk, since it always contained less casein and more globulin. At times the fat content approached normal but the non-fatty solids were usually deficient. These results differ from those

previously reported with goats, in that in goats the treatment induced the secretion of large amounts of normal milk.

We are indebted to Dr F. L. Pyman, F.R.S., of Messrs Boots Pure Drug Co., Ltd., for the diethylstilboestrol used in these experiments and to the Agricultural Research Council for a grant in support of this work.

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The effect of extravascular suffusion of blood and of the experimental introduction of blood and plasma on capillary resistance. By HAROLD SCARBOROUGH. (*From The Clinical Laboratories, The Royal Infirmary, Edinburgh*)

The functions of the capillary walls in man are not readily susceptible to investigation. Determination of the capillary resistance, however, affords a simple and, provided certain standard conditions are observed, a relatively accurate method for investigating one of their properties. Upon somewhat unsatisfactory evidence it is at present widely held that in otherwise healthy subjects a low capillary resistance is associated with an inadequate intake of ascorbic acid in the diet. It is surprising, therefore, that such conflicting results have been obtained in scurvy, both high and low values having been reported [Hess & Fish, 1914; Goettsch, 1935; Weld, 1936; Scarborough, 1940].

The explanation for this discrepancy is that the introduction of more than about 4 c.c. of blood into the tissues produces within 12 hr. a 50-100% increase in capillary resistance which is maintained for some 2-4 days. The introduction of blood spontaneously or experimentally into the subcutaneous tissues, muscles, alimentary tract or veins produces this effect. The phenomenon has been obtained with both 'normal' and scorbutic blood, with blood stored for 6 months, with cells alone and with plasma alone, but not with saline or with glucose-saline. The intramuscular injection of plasma produces a slight effect, but the response is marked after the intravenous injection of plasma or a 50:50 plasma-saline mixture. The increase in capillary resistance is observed after haemorrhage in scurvy and in certain of the bleeding diseases as well as after surgical operations. Due allowance must be made for this phenomenon in investigating these conditions.

The significance of these results is not at present clear. We have obtained evidence that a bleeding tendency is diminished during the period after haemorrhage into the tissues, while the capillary resistance

remains increased. These results acquire fresh interest from the recent observations of Macfarlane [1941] on the importance of the part played by capillaries in the mechanism of haemostasis.

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Visual responses in the cat and monkey. By E. D. ADRIAN

In the monkey (under dial or nembutal) illuminating the eye gives a discharge of impulses to the visual area of the cortex which rises to an initial peak and then declines gradually, continuing for 10 sec. or more if the light is maintained. In the cat a very feeble light gives a continued discharge to the visual area, but a bright light gives an initial outburst followed by an abrupt cessation of the discharge. A sheet of white paper reflecting daylight is usually enough to produce this result in the cat, whereas in the monkey there has been no sign of it even with light of dazzling intensity (the beam of a 100 c.p. flood light). When darkness is restored there is a brief 'off' discharge to the cortex in both animals, but in the monkey there is very little activity in the dark, whereas in the cat a persistent discharge follows the 'off' effect. There is often a gradual increase in the intensity of this discharge, owing, probably, to dark adaptation.

These differences between the cat and monkey are due to the retinal rather than the cerebral mechanism, for they are found in the optic nerve response as well as in the discharge reaching the striate area. They are not confined to certain regions of the striate area or retina, though detailed comparison of the response in different regions has not been attempted. In deep anaesthesia the response in both animals is reduced to the brief 'on' or 'off' discharge: the difference only becomes obvious when the level of activity is higher. Inhibitory processes in the retina have been described by Granit [1938] and by Hartline [1938], and no doubt they occur in the monkey's eye as well as in the cat's, but there seems to be nothing in the monkey to correspond with the complete cessation of activity in the cat's eye exposed to a bright light.

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Galactose-1-phosphoric acid in galactose metabolism. By H. W. KOSTERLITZ and C. M. RITCHIE. (*From the Department of Physiology, Marischal College, Aberdeen*)

In a previous paper [Kosterlitz, 1939] galactose-1-phosphoric acid was shown to be an intermediary in the fermentation of galactose by galactose-adapted *S. cerevisiae* Froberg. The estimation of the rate of acid hydrolysis and of the optical activity of the impure galactose-phosphate from the livers of rabbits assimilating galactose, made it probable that this galactosephosphate was identical with synthetic galactose-1-phosphoric acid.

Further investigations on the role of galactose-1-phosphoric acid show that:

(1) Minced liver or liver extracts do not dephosphorylate galactose-1-phosphoric acid as easily as they do glucose-1-phosphoric acid [see also Cori, Colowick & Cori, 1938]. At room temperature, galactose-1-phosphoric acid resists the action of minced liver or liver extract for 120 min. During 180 min. at 37.5° C. one-third to one-half of the ester was dephosphorylated by minced liver, and 10–13% by undialysed or dialysed liver extracts.

(2) Galactose-1-phosphoric acid is a stronger acid than is phosphoric acid. Galactose-1-phosphoric acid has pK_1 1.00 and pK_2 6.17, while phosphoric acid has pK_1 1.95 and pK_2 6.84. Thus, formation and storage of the ester in the liver may cause an apparent increase in the respiratory quotient. The storage of 1 mol. of ester would cause the appearance of 0.3–0.4 mol. of extra CO_2 .

(3) The rapid fermentation of galactose-1-phosphoric acid by dried galactose-adapted *S. cerevisiae* Froberg was absent when the same strain of yeast was grown on sucrose instead of on galactose. In this case, neither ester nor free galactose was fermented. With living galactose-adapted *S. cerevisiae*, the rates of fermentation decreased in the following order: glucose, galactose, galactose-1-phosphoric acid and, finally, glucose-1-phosphoric acid. The slow rate of fermentation of the esters was probably due to the cell membrane being less permeable to the esters than to the free sugars.

An expenses grant by the Medical Research Council is gratefully acknowledged.

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Activity in visceral nerves. By W. BURNS. (*From the Department of Physiology, Marischal College, Aberdeen*)

An examination of the action potentials in the sympathetic nerves of the alimentary canal and associated structures in the frog has confirmed the occurrence of the three types of impulses described by Tower [1933]. Recording from the caudal end of a cut ramus intercommunicans or the peripheral end of a divided grey and white ramus (these are usually fused together in the frog), the three main types of potential distinguishable are: (1) Fast impulses similar to those in cutaneous nerves. These can be elicited by light touch or especially by stretching of vascular mesenteric areas. Their amplitude in monophasic records is approximately $90 \mu V$. (2) Slower impulses resulting particularly from fairly heavy pressure applied almost anywhere on the gut or mesenteric area and in amplitude $30-50 \mu V$. (3) Slow potential changes or waves lasting $0.015-0.03$ sec. which occur spontaneously and in response to various stimuli, e.g. localized burning with acid, which increases the amplitude and frequency. These may be of $20-80 \mu V$. or more in amplitude. They may occur spontaneously in trains of regularly occurring potential changes at a frequency of about 15 per sec. All three types of impulses occur in isolated gut preparations and in the spinal frog. Types 1 and 2 appear to be afferent in nature but type 3 is difficult to define; an effort to elucidate their nature has resulted in the following findings: (1) Waves occur in the cranial end of a cut ramus intercommunicans as well as in the caudal end. (2) The fused grey and white ramus is cut, and a record, obtained from the end not connected to the ganglia of the sympathetic chain, shows slow impulses which appear to be increased in amplitude and frequency by injuring the gut with acid, a stimulus which also causes increased wave formation. (3) The waves do not appear spontaneously or in response to stimuli in a record from a posterior spinal nerve root, whereas the fast impulses do. It seems possible, therefore, as suggested by Tower, that the slow impulses may represent efferent activity of some sort.

Expenses grants from the Medical Research Council and the Government Grants Committee of the Royal Society are gratefully acknowledged.

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An optical device for recording small changes of pressure or volume. By J. ADLER, G. H. BELL and J. A. C. KNOX. (*From the Institute of Physiology, University of Glasgow*)

This apparatus was designed to record the very small movements of the cervix of the uterus in vivo. A small rubber balloon tied to the end of a cannula is placed in the cervical canal and connected by tubing to the device of Fig. 1 made of Perspex or glass. The system is filled with water so that the contractions and relaxations of the cervix produce changes in the level of the water in the space enclosed between the two

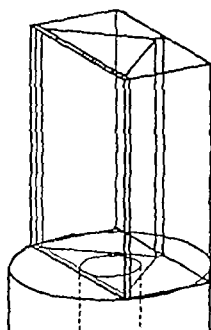


Fig. 1.

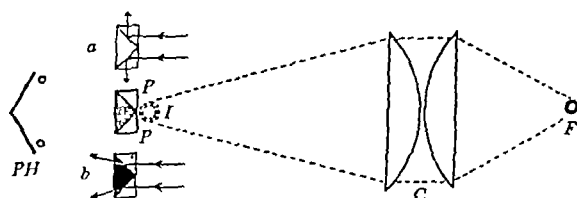


Fig. 2. Diagrammatic scheme of apparatus viewed from above. *F*, filament of 10 V. 7.5 amp. lamp; *C*, condenser; *I*, image of *F*; *PH*, photocell; shaded area is prismatic space enclosed by prisms *P* and *P'*; *a*, path of light when space is filled with air; *b*, path of light when space is filled with water.

prisms and the glass cover plate. An image of the filament of a motor car lamp is projected on to the surface of the prisms (Fig. 2). When there is no water in the shaded area the light is reflected at the oblique surfaces (Fig. 2*a*), but when the water rises light passes through to the photocell (Fig. 2*b*). The higher the fluid stands in the prismatic space within the range of the image of the filament the greater the illumination of the photocell. The movements of the fluid are recorded on smoked paper by the method already described [Bell, Bell, Knox & Smellie, 1937].

The sensitivity of the apparatus varies inversely as the cross-section of the prismatic space and the size of the projected image. The divergence of the transmitted beam can be reduced and better illumination of the photocell secured by using a fluid with a refractive index higher than that of the material of the prisms.

In the apparatus demonstrated a movement of 0.01 c.c. gives a deflection of 1.0 cm. on the kymograph drum.

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Economical development of long lengths of film or bromide paper. By W. BURNS. (*From the Department of Physiology, Marischal College, Aberdeen*)

The apparatus described was evolved, in the apparent absence of anything suitable on the market, to fulfil these requirements: (1) lengths of film or bromide paper 30 ft. or more long must be developed using the minimum amount of developer; (2) the apparatus must be simple and inexpensive to construct. These conditions are fulfilled by the following arrangement. A cylindrical wooden framework or drum made of two discs of wood 12 in. in diameter joined by wooden slats 18 in. long is mounted on a brass spindle, and on this the strip of film or paper is rolled, emulsion outward, and secured by small phosphor bronze clips at the ends. If paper is used, preliminary moistening to minimize subsequent stretching and loosening is desirable. The drum is then placed on a stand and rotated over a shallow tray 20 in. long, 10 in. wide and 1 in. deep containing developer. The stand is arranged so that the minimum volume of developer is needed to wet the film or paper. In the present arrangement this volume is about 1000 c.c. The drum is then lifted off and washed by rotating a few times in water, and then put on a second identical stand with fixing solution in its tray. The wood of the stands and drum is treated with an aniline-bichromate finish which is resistant to alkali and acid, and the spindle is chromium plated to prevent corrosion. The trays used are standard enamelled metal trays sold for domestic purposes. Rotation of the drum by hand is quite satisfactory, but a motor drive could be easily arranged if desired.

PROCEEDINGS
OF THE
PHYSIOLOGICAL SOCIETY
1 October 1941

The effect of acid and alkali on muscle vessels.

By R. J. S. McDOWALL. *King's College, London*

It has been shown [McDowall, 1928] that it is possible to produce in perfused muscle vessels an acid and alkaline tone which is relaxed by dilute alkali or dilute acid respectively. The injection of acid and alkali into the intact circulation has remarkably little effect, but this is not wholly due to the buffering of the blood. There is also a balancing of the indirect effect on vessels through the vasoconstrictor centre and the direct effect on the vessels.

Thus, if lactic acid is injected in amounts just large enough to lower the pH of the blood, there is little or no sustained change in the blood pressure or the volume of a skinned limb, but if the sympathetic to the part is cut there is an abnormal increase in the volume of the limb, and this may be caused to return to normal in steps by the injection of sodium bicarbonate. The peripheral effects of the alkali are abolished by deep anaesthesia.

It is found also that alkalization reverses or abolishes the vasodilatation usually produced by minute doses of adrenaline.

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PROCEEDINGS

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15 November 1941

Physiology of sudden expiration. By J. SKLÁDAL
(introduced by E. D. ADRIAN), *Prague*

The physiology of sudden expiration is quite different from that of quiet expiration. It is represented by four phases instead of the normal one, three of which are passive and provoked by the active expiratory movement. The cause of this splitting of the air wave is the glottal narrowing, which in suddenly performed expiration becomes a real obstacle to the continuous flow of the expired air. This is proved by the aerotachography of this air wave in sudden expiration, by the oscillographic curve of the so-called sound of expiratory reduplication recorded in sudden expiration, experimental reconstruction of the reduplicated sound performed by means of a metal syringe on lungs extracted from large domestic animals, as also by the simultaneous auscultatory and radioscopical examination in patients with the so-called gallop rhythm of pulmonary sounds in sudden expiration.

(1) The aerotachographic curve of a simple sudden expiration shows two waves.

(2) On the oscillogram of patients in whom instead of one, two expiratory sounds could be heard, two groups of oscillations interrupted by an interval can be seen.

(3) By an artificially provoked sudden expiration on isolated animal lungs two sounds will be heard over the trachea and over the great bronchi as also over the peripheral area of lungs which has been consolidated by chemical action. On removal of the larynx, only one sound is heard over the same area, thus proving that the cause of reduplication must be sought in the glottal narrowing.

(4) A very simple physical proof of the importance of the rima glottidis is given by the following experiment with the metal syringe of

a content 200 c.c. of air: exercising a sudden aspiration with the piston of the syringe two sounds are heard in the case of a narrow (lumen 2 mm.) opening. Remaking the same experiment with a large opening (lumen 20 mm.) of the syringe, only one sound is perceived.

(5) In certain patients, by means of sudden expiration, the so-called pulmonary gallop with three breath sounds can be heard. Here, simultaneous auscultatory and X-ray screen examination prove that the third sound is aspiratory, and consequently has an opposite direction to the two preceding expiratory waves.

PROCEEDINGS

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PHYSIOLOGICAL SOCIETY

14 February 1942

The potentiation of adrenaline dilatation by histamine.

By R. J. S. McDOWALL. (*King's College, London*)

Adrenaline dilatation of muscle vessels has been shown to vary very much according to the circumstances of the experiment, especially in regard to the reaction of the blood and the degree of central nervous activity maintaining.

It is now found that in many animals a previous intravenous injection of histamine greatly enhances the dilator effects of adrenaline on muscle vessels, although all the other effects of the histamine have apparently passed off.

It is suggested that if histamine is liberated in muscular exercise it may play a part in determining the relative amounts of dilatation and constriction produced by adrenaline on muscle vessels.

The influence of amino-acids on transfer of phosphate in muscle extract and on the solubility of Mg^{++} and Ca^{++} salts. By H. LEHMANN (*Beit Memorial Fellow*) and L. POLLAK. (*Biochemical Laboratory, Cambridge*)

Amino-acids accelerate enzyme reactions responsible for phosphate transfer in muscle extract. They also increase the solubility of magnesium phosphate, and this change in solubility may partly explain the effects found in muscle extracts. These observations led us further to investigate the solubility of calcium salts in the presence of amino-acids.

A. *Muscle extracts.* Extracts from skeletal muscles of rabbits and crabs, prepared according to Meyerhof, were dialysed for several days, made alkaline and activated by Mg^{++} . The addition to these extracts of α -amino-acids, particularly of those containing an SH group, initiated or accelerated (without changing the final equilibrium) the transfer of

phosphate from adenylypyrophosphate to creatine or to arginine and from adenosine diphosphate to creatine. It also accelerated the change of 3-phosphoglyceric acid into 2-phosphopyruvic acid. The same effect was obtained with thioglycollic acid.

B. *Solubility of Mg^{++} and Ca^{++} .* The solubility of magnesium salts of inorganic phosphorus and certain phosphoric esters was increased by the same compounds which activated the transfer of phosphate. The effect was not specific for magnesium phosphate, but could also be observed with magnesium carbonate and calcium phosphate.

Solubility of $CaHPO_4 \cdot 2H_2O$ in mg. % at room temperature

Neutral pH		pH 8.5. Glycine buffer (9 mol. glycine : 1 mol. NaOH)	
		Concentration of buffer	
Water	13	M/10	11
0.6% glycine	16	M/5	14
1.3% glycine	20	M/2	25
5.0% glycine	36	M/1	30
0.6% cysteine	20		
1.3% cysteine	33		

The two kinds of experiments *in vitro* might be related to processes *in vivo* as follows:

A. The breakdown of carbohydrate *in vivo* is known to involve phosphorylations and is also activated by protein breakdown. However, to obtain a clear-cut effect of amino-acids on phosphate transfer in Mg^{++} activated dialysed muscle extract, a pH is needed which is greater than that occurring *in vivo*.

B. The increased solubility of calcium salts in the presence of α -amino-acids even in alkaline medium may have a bearing on the absorption of calcium in the intestine and on calcium therapy. Even soluble calcium preparations are bound, once they are administered, to form some carbonates and phosphates which in their turn could be rendered more soluble by amino-acids.

One of us (H. L.) is indebted to the Ella Sachs-Plotz foundation for a grant towards research expenses.

A haemoglobin-saline perfusion solution.

By D. H. SMYTH. (*University College, London*)

Amberson & Höber [1932] described a haemoglobin-saline solution for perfusion experiments. Brown & Dale [1936] reported favourably on its use, and found also that such a solution could be sterilized by passing through a Seitz filter, provided that the cell debris be completely removed

by use of the Sharples centrifuge, the ordinary methods of filtration and centrifuging being unsatisfactory for the purpose. A method is described here of preparing a haemoglobin solution which will pass through a Seitz filter without the use of a Sharples centrifuge, as this is often not available.

Washed red cells of the ox are laked by the addition of distilled water in the proportion of 75 ml. of distilled water to 15 ml. of cells, and the solution is filtered. It is then shaken with about one-fifth of its volume of ethyl ether. The emulsion is centrifuged and the upper ethereal layer discarded. The aqueous haemoglobin solution is filtered, and the ether is removed by drawing a current of air through at low pressure and 40° C. The frothing which occurs during this procedure results in a small loss but is otherwise harmless. The solution is again filtered and will now pass easily through the Seitz filter. The salts can be added either before or after sterilization.

The adequacy of this solution for perfusion experiments is shown by the fact that it will maintain the beat of the isolated rat's heart, and it has also been used successfully as the perfusion solution in a rat's heart-lung preparation, prepared by Mr R. A. Gregory. After perfusing, the solution can be sterilized and used again in a subsequent experiment.

The treatment of the solution with ether also seems to cause some reduction in the tendency to frothing, a property especially valuable where artificial oxygenators are used.

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Growth and pituitary diabetogenesis. By F. G. YOUNG. (National Institute for Medical Research, Hampstead)

The suggestion was recently made [Young, 1941] that the excessive weight of many adult human diabetics, as well as the rise in body weight observed in dogs and cats effectively treated with diabetogenic pituitary extracts, might result from an increase in anterior pituitary action, balanced, for a time at least, by increased activity of the pancreatic islets. The rise in body weight might therefore be regarded as a protective reaction, whereby the carbon of the potential carbohydrate, of which the oxidation is depressed by pituitary over-action, is stored in a stable form.

Diabetes develops when the islets of Langerhans are unable to maintain their necessarily increased activity, or any further increase in the storage capacity of the tissues is impossible.

It is possible to demonstrate the growth-promoting action of pituitary extract in an animal receiving a constant daily amount of food, which, before pituitary treatment began, just maintained a constant body weight. If a dog or a cat is thus treated with diabetogenic pituitary extract, the body weight may rise and nitrogen be retained, despite the appearance of glycosuria and the absence of extra food. Nevertheless, the appetite is increased under such conditions, and when extra food is given after a period of pituitary treatment with constant and (normally) adequate food intake, the body weight may again rise. This delayed response might be expected if the growth-promoting action of pituitary extract is associated with hyperplasia of the pancreatic islets. Clinically, an increase in food intake may not be essential for a prediabetic rise in body weight, although an increase is perhaps more probable. Diabetes may be considered as a disease associated not only with over-nutrition, but also with attempted excessive retention of a normal food intake.

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Circulation in the placenta of the sheep. By J. BAROROFF and D. H. BARRON. (*Physiological Laboratory, Cambridge*)

(1) Sheep possess a cotyledonous placenta. The weight of the cotyledons is greatest about the twelfth of the 21 weeks of gestation. Research was undertaken to investigate the structural basis of this early development. It is due to the formation of great masses of foetal tissue histologically similar to the Wharton's jelly of the cord.

(2) The relation of these masses to the foetal vascular system is as follows. The substance of the masses is non-vascular except for a few axial vessels. Often only one axial vessel is seen in a mass. Spread out over the surface of the mass is a close reticulum of foetal capillaries which in turn is just below the foetal-maternal membranes. The vessels in the maternal tissue on the other hand permeate the whole tissue. The contact between the foetal blood and the maternal is the closest of which morphological structure permits. The foetal blood, it will be recalled, attains upwards of 90% oxygen saturation at this age.

(3) From about the fourteenth week onwards the masses tend to disappear, leaving their vascular coverings. It is uncertain to what extent the jelly becomes organized and to what extent it becomes absorbed; in favour of the latter explanation is (i) the loss of weight of the cotyledons, and (ii) the constancy after this date of the volume of foetal blood therein. These coverings become folded and corrugated, and at this stage the degree of separation of the foetal from the maternal vessels is in space very small.

(4) Towards term the foetal and maternal vascular elements tend to sort themselves out into ropes of vascular tissue. The contact is, over much of the placenta, at that stage between the ropes rather than the individual vessels, an arrangement by no means so favourable to the exchange of materials. Actually at this stage the blood of the umbilical vein is much less completely oxygenated than earlier.

We should like to thank Miss M. E. Carsten who sectioned the tissues, and the Rockefeller Foundation which defrayed the expenses of the research.